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A Publication of the American Veterinary Medical Association

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VOL. XVIII JANUARY, 1957 No. 66

The Diagnosis of Squamous Cell Carcinoma of the Eye (Cancer Eye) in Cattle

A. W. MONLUX, D.V.M., Ph.D.; W. A. ANDERSON, D.V.M., M.S.; C. L. DAVIS, D.V.M.

Denver, Colorado

IN THE STUDY of epithelial tumors of the orbital region in cattle, one must accept that benign and malignant tumors will be encountered. It has been established that many, but not all, of the benign growths, if left untreated, will undergo a transformation into carcinoma. Some growths are reported to spontaneously regress and a few to completely disappear. Little attention has been given in the past to the differences in behavior between carcinoma of the eyeball and those arising in the eyelids and adjacent skin. Some veterinarians, particularly in the West where the disease is more frequently encountered, have become quite adept at differentiating by visual examination between benign, malignant, and inflammatory conditions of the orbital region which include or are confused with classic squamous cell carcinomas.

This paper illustrates the gross and microscopic morphology of some of the more controversial lesions encountered in 548 cattle with diseased eyes or circumocular tissues. Types of epithelial tumors from these same anatomic sites which can be regarded unequivocally as either benign or malignant tumors are also discussed. Of these 548 cattle, 532 had one or more squamous cell tumors of the orbital region and represented the bovine ocular tumors reported in a recent survey.[19]

Epithelioma is not used as a synonym for squamous cell carcinoma of the bovine eye in this article as it is obsolete and is increasingly being erroneously interpreted in reference to cattle to include benign squamous cell tumors by not only many in the veterinary profession but by those in closely allied medical sciences. Epithelioma as a name for squamous cell carcinomas of the bovine eye replaced the misnomer "fungus haematodes" (bleeding fungus) early in this century following the advent of the doctrine of cellular pathology and subsequent investigations which proved that the lesions were of neoplastic origin. Feldman,[11] Runnells,[20] Kral and Novac,[16] and other veterinary writers preferred to classify malignant epithelial tumors of the bovine eye as carcinomas rather than epitheliomas. Jackson,[14] in 1936, in his study of tumors of domesticated animals, used epithelioma as well as acanthoma as synonyms for a malignant tumor of a squamous type of epithelium but admitted that such a classification was confusing. He stated that certain benign or not clearly malignant neoplasms which arise from the accessory epidermal structures are often also grouped as epitheliomas.

Even more confusion about the use of the term epithelioma exists in the classifi-

From the Animal Disease and Parasite Research Branch Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Denver Federal Center, Denver, Colo. Dr. Davis is now with the Animal Disease Eradication Branch, ARS, U.S.D.A., Washington, D. C., and Dr. Monlux is with the School of Veterinary Medicine, Oklahoma A. & M. College, Stillwater.

The authors are indebted to Dr. J. W. Hovorka, inspector-in-charge; Dr. A. L. Stigers, assistant inspector-in-charge (now inspector-in-charge, Meat Inspection Branch, ARS, U.S.D.A., Dubuque, Iowa); and to the veterinarians assigned to the Meat Inspection Branch, ARS, U.S.D.A., Denver, for their cooperation with veterinarians at the Animal Disease Research Laboratory in the collection of specimens. All of the tumors reported in this survey were detected in routine meat inspection procedures by those veterinarians.

cation of human tumors. Various texts which discussed the diseases of man continued to use epithelioma as a synonym for squamous cell carcinoma of the skin as recently as the last decade. An "Atlas of Dermal Pathology,"[5] sponsored by the National Research Council in 1947, described malignant tumors of the human skin as epitheliomas. Most of the recent texts on human pathology which discuss neoplastic diseases[1,29] have abandoned the use of the term epithelioma to describe malignant tumors. There is a growing tendency in the study of human tumors for the word to indicate only the noninvasive basal cell carcinomas[1] such as the Brooke tumor.

Day,[9] in 1932, probably perpetuated the use of epithelioma in veterinary medicine by his observation that since the bovine orbital growths were epidermoid carcinomas, the older term, epithelioma, was still appropriate. Bengston,[7] in 1938, associated epithelioma with carcinoma when he stated "microscopic examination of small, early growths show definitely that they are malignant carcinomatous new growths or tumors." Creech[24] described epitheliomas in 1942 as cancerous or malignant types of tumor. It can be assumed from the observations of these three veterinarians, who were primarily engaged in the examination and diagnosis of specimens collected from diseased animals by the Meat Inspection Service, U.S.D.A., that the terms cancer eye, squamous cell carcinoma, and epithelioma were interchangeable and had the same meaning to them.

Recognition of malignancy, or associated objectionable secondary changes, or both are obviously the important considerations in evaluating these ocular tumors in meat inspection. However, there are many veterinarians engaged in various phases of clinical practice who might consider modifications of their treatment of some of the bovine eye tumors to either a more or less radical procedure if they were aware of the appearance of benign growths and the early and more advanced carcinomas of the bovine eye.

The great economic waste and the justification for antemortem condemnations by federal meat inspectors of animals with advanced cases of "epithelioma of the eye" were the subject of an editorial[10] in the *Journal of the American Veterinary Medical Association* in 1947.

Steiner and Bengston in their discussion of carcinoma of the eye in cattle[23] stated that most cases are found in Hereford cattle. We believe that, as an economic problem, the disease is of importance only in the Hereford breed. Reports in the literature of cases in other species (we report several in this survey) have been misconstrued by a few writers in recent years as an indication that the problem is equally as great in other species; this is simply not true.

Of the numerous other publications on the general subject of carcinoma of the bovine eye, the reader is referred to several[6,8,12,13,15,17,18,21,25-28,30,31] for additional information.

COLLECTION OF DATA

As a part of the survey and collection of bovine tumors, this laboratory requested the Federal Meat Inspection Service, Denver, Colo., to save, during the calendar year 1953, all lesions involving the eyeball, eyelids, or adjacent skin which either might be, or could reasonably be confused with, tumors of the eye. Eye lesions were actually systematically collected in only three of the larger Denver abattoirs as inadequate manpower and plant facilities made a complete collection inadvisable. However, we believe a reasonably accurate cross section or sample of cases was obtained.

A total of 3,079 cases of epithelioma was reported in the inspection procedures at Denver in the year of the survey (table 5); which indicates the enormity of the task if an attempt were made to collect all malignant eye tumors in addition to the benign growths for one or two years.

The reasons for extending the collection over at least one complete calendar year are discussed in the article[19] on the occurrence of neoplastic diseases in slaughtered, food-producing animals. That article evaluates these same tumors in relation to other neoplasms of cattle. The actual monthly variations in the submitting of carcinomas of the eye to this laboratory (table 3) can be taken as an approximation of the proportional monthly occurrence which might be encountered in Denver abattoirs. Our data do not include an evaluation of the necropsy diagnosis of squamous cell carcinoma of the eye.

Before the collection of ocular tumors began, serious consideration was given to the type of data which should be recorded. During 1952, we made preliminary observations on a limited number of cases in slaughtered cattle and reviewed many of the cases of bovine carcinoma of the eye in the Denver Animal Disease Research Laboratory files. This evaluation allowed us to make maximum use of specimens and shortened the reports which were necessary to properly identify each submitted tumor.

Data on each bovine case were systematically recorded on 5- by 8-inch cards to make the data accessible for review. Data for the tables were compiled from these cards. Exact three-dimensional measurements to the nearest millimeter were made on every tumor located on the eyeball, eyelids, or periocular skin. Multiple tumors frequently occurred in these sites and, consequently, measurements, anatomic location, and general descriptions were made of each individual tumor encountered (even if an eye was involved with 4 or 5 distinct growths). Bilateral involvement was no problem in collecting data as each eye (eyeball, eyelid, and periocular skin) was considered as a single unit.

An attempt was made to study the specimens during the day on which the animal was slaughtered. If there were eyelid lesions, the complete eye including periocular skin was sent to the laboratory. At times, when the lesions were limited to the cornea and bulbar conjunctiva, only the eyeball (usually including the membrana nictitans for orientation) was submitted. The collection of periocular skin and eyelids was done only when it did not interfere with the slaughtering or inspection processes. Specimens were examined and all pertinent data recorded before fixing them in 10 per cent formalin.

Gross photographs were made of typical as well as unusual eye lesions. At least one paraffin section was made of each lesion and these were stained routinely with hematoxylin and eosin for microscopic examination. Data were also recorded and tissue preparations made on all associated metastatic lesions. Many hyperplastic parotid and cervical lymph nodes were studied microscopically in an attempt to find early metastatic lesions.

Only preliminary studies[18, 18, 21, 25, 30, 31] were found in the literature regarding the relation of periocular skin pigmentation to bovine carcinoma of the eye. The majority of investigators believe that Hereford cattle with eyes surrounded by white hair are more likely to develop carcinoma of the eyes than those whose periocular skin contains red hairs. Some breeders select breeding animals with red hair around the eye for that reason. The location and degree of pigmentation necessary to produce a desirable effect are important considerations in these breeding programs. Lush[30] mentioned the more frequent "snow blindness" in white-eyed Herefords than in red-eyed ones in Wyoming and believed that brilliant sunlight was the most important contributing factor. He interpreted this as an indication that brilliant sunshine also could predispose to ocular tumors.

It is impossible, under the limitations of our collection, to record the degree of pigmentation of the skin around affected eyes on individual animals. Observations on pigmentation would have had to be made before slaughter to be accurate. An attempt was made only to determine what problems might be encountered in evaluating degrees of pigmentation.

The periocular skin was not submitted in many

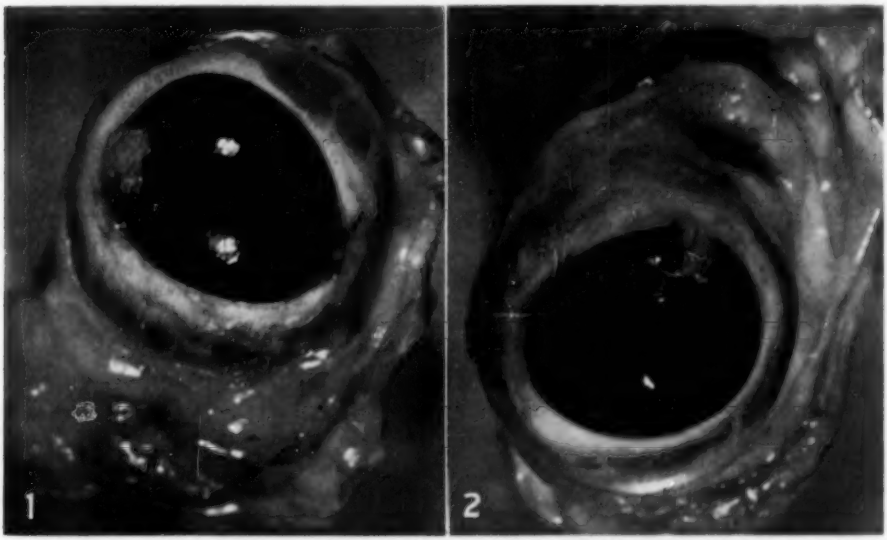


Fig. 1—An epidermal plaque on the eye of a 7-year-old Hereford cow. It covered a 10- by 8-mm. area on the cornea and bulbar conjunctiva. Figures 14 and 15 are photomicrographs of this same lesion (case D 2202).

Fig. 2—An epidermal papilloma of the eye in a 6-year-old Hereford cow. The tumor had a 3- by 3-mm. base at the limbus and some of the papillary projections from this point of attachment were up to 10 mm. in length (case 131336).

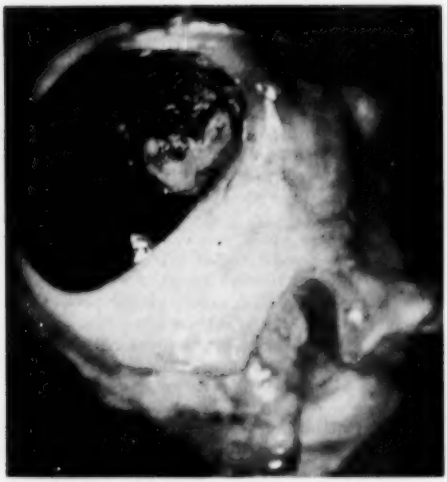


Fig. 3—An epidermal papilloma (15 by 10 by 4 mm.) of the eye in a 6-year-old Guernsey cow. Figures 17 and 18 are photomicrographs of this same lesion (case D 2289).

of the cases where lesions were seen only on the eyeball. In addition, segments of the eyelid and skin might have been missing or distorted in the recovery of lesions in the slaughtering process. However, the greatest deterrent to the evaluation of pigmentation at necropsy was that any existing pigmentation of the periocular skin could have been easily masked by tumors on the mucocutaneous junction of the eyelid, by their spread in the skin, or by the frequent secondary ulceration. An evaluation of periocular skin pigmentation should be included in current studies on the developmental growth of bovine ocular carcinomas in selected herds of Hereford cattle. Such projects will be extremely valuable in establishing the behavior of lesions designated as benign or precancerous in this article.

Hereford cattle in Colorado, including feeders from other states, appear to be bred for a consistent white face. Few completely red-eyed Herefords (1 or both eyes having an unbroken band of red hair completely encircling the eye) were observed in the stockyards. Within some of these complete bands and the more frequently encountered partial bands, small areas of white hair were frequently included adjacent to the eyelids and often only a comparatively small portion of the actual eyelid contained red hair. Longer white hairs were usually intermixed in the pigmented areas in these partially pigmented eyelids.

A few of both the completely white-faced Herefords (all hair was white around both eyes) and those with varying degrees of pigmented periocular skin had small, light brownish areas of skin bordered by unquestionable white skin in the periocular skin and the skin of the eyelid. White hairs arose from these areas which may be a possible intermediate degree of pigmentation. It is necessary to thoroughly wash eyelids to detect this questionable type of pigmentation since tear stains and conjunctival exudates will produce a discoloration which resembles it.

The degree of lacrimation and conjunctivitis was much more marked in the nonpigmented groups observed in the stockyards and there was a definite glare effect around these white eyes in bright sunlight. Ocular exudation and conjunctivitis decreased noticeably when red hair arose from one half or more of the border of periocular skin. Shipping and handling of these animals may have accentuated the conjunctivitis as compared to that encountered under average range conditions.

DIAGNOSTIC CRITERIA

A histological examination is often necessary in the study of inflammatory and neoplastic conditions of the eye to confirm or refute clinical or necropsy impressions or diagnoses. Adoption of elaborate classifications based on pathological or clinical features should be avoided. Errors in the interpretation of individual lesions are occasionally made under any system of classification regardless of whether the system be simple or complex. Designations of papilloma, plaque, early squamous cell carcinoma, and squamous cell carcinoma have value to both the clinician and pathologist in understanding the bovine epithelial ocular tumors. The introduction of a more detailed classification would be cumbersome.

Papillomas and plaques (fig. 1) represent benign squamous cell tumors and have a slightly different behavior which might be important in evaluating clinical studies. Ocular papillomas are epithelial outgrowths from stratified epithelial structures of the skin or mucous membranes. Classically, there is an actual folding of the epithelium to form inner cores of connective tissue and superficial layers of proliferating epithelium. Several types of papillomas were seen. The most unmistakable papillomas were the lesions with one or more long, frondlike branching structures (fig. 2). Small papillary projections arose from the secondary divisions of this type of papilloma which was characteristically attached by a small base. At the other extreme, there were tumor masses consisting of many small rounded protuberances which had a large, confluent basal area of attachment to the eye (fig. 3).

However, most of the papillomas were represented by an intermediate type of growth. They usually had a broad base and multiple, hard, spinelike projections of variable sizes. A few cauliflower or mushroom-like papillary tumors, attached by a narrow stalk to the eyeball, were also seen.

Many of the large squamous cell carcinomas had this same papillary structure. However, there was dyskeratosis throughout all of these tumors and changes characteristic of an invasive carcinoma at the base of the stalk (fig. 4 and 5). They invariably protruded through the palpebral fissure and were often so large as to cause an open fixation of the eyelids (fig. 6).

In 1952, several veterinarians engaged

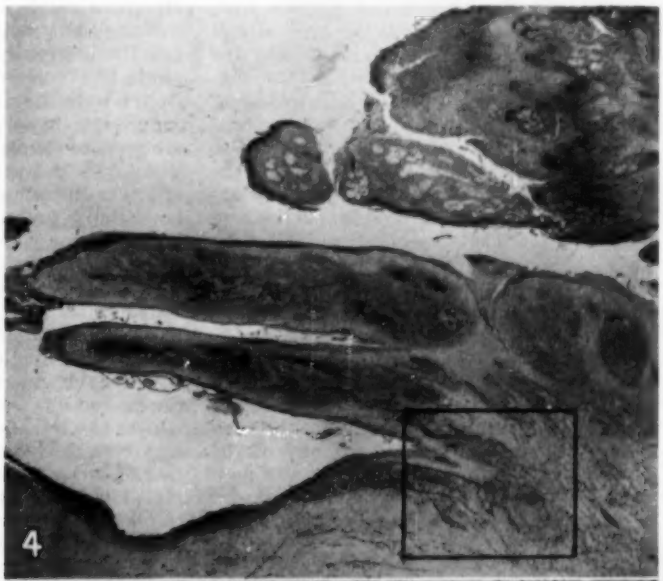


Fig. 4—A border of a papillary squamous cell carcinoma of the eye of an old Hereford cow. A higher magnification of the blocked area is shown in figure 5 and is one of several points of transformation of the tumor into frank squamous cell carcinoma (case 131565). Hematoxylin-eosin stain. x 20.

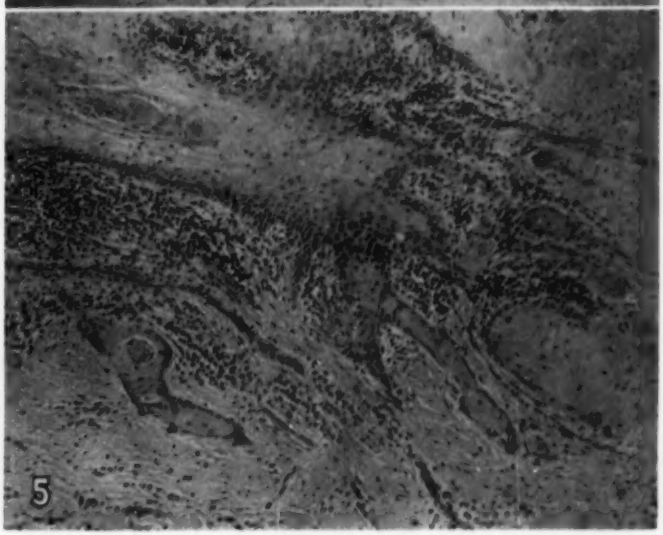


Fig. 5—An invasive portion of the tumor shown in a block in figure 4. Note the nests of tumor cells breaking away from the basal cell layer of the tumefied epithelium on the right side. Changes in the basal cell layer, also seen in the lower central portion, were consistent with what was regarded as early carcinoma (case 131565). Hematoxylin-eosin stain. x 95.

in federal meat inspection at Denver used the terms "plaques" and "papillomas" to describe two types of benign eye tumors. Most plaques were easily separable from papillomas. As observed by the inspectors, the plaques were gray to white, rounded, slightly raised growths with flattened surfaces. They were usually opaque and had either a smooth, granular, or slightly roughened exterior. Smaller plaques and a few of the large extensive plaques were occasionally filmlike, smooth, and almost transparent. While the common site for plaques is on the cornea and bulbar conjunctiva with most of the lesions involving the junction zone between the bulbar conjunctiva and cornea (limbus), they also appear on the skin of the eyelid and the palpebral conjunctiva adjacent to its cutaneous junction. The plaques originating on the limbus have a tendency to extend a greater distance over the cornea than over the conjunctiva (fig. 1). This is not surprising if the more uniform smoothness of the cornea is considered.

A plaque is described in most dictionaries as resembling a coin, patch, or blood platelet and this is a reasonably accurate general description of the tumors we have grouped under that term. Tumors of the human eye similar to our plaques and papillomas have been extensively described by Ash [1,2] and Ash and Wilder [3,4] in their studies of the collection of eye tumors at the Armed Forces Institute of Pathology at Washington, D. C. This collection of tumors of the human eye is undoubtedly the largest in the world. Their description of epidermalized or metaplastic epithelium forming papillomas and leukoplakic or Bowen's type of dyskeratosis is almost identical to our experiences with the bovine eye tumors. However, these lesions in man are rare compared to the reported high occurrence of conjunctival tumors in Hereford cattle.

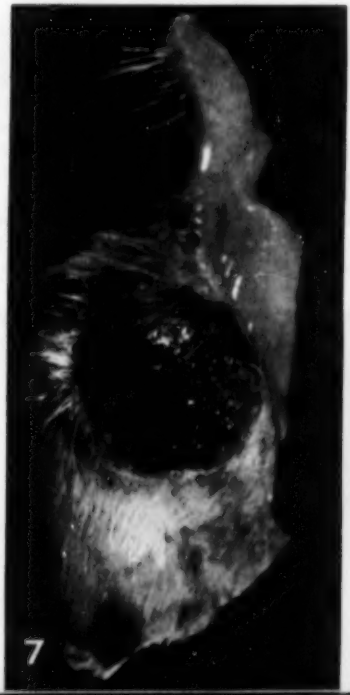
Since the submitted bovine plaques were usually intermediate between the benign, senile thickenings of the epidermis seen in Bowen's disease in man and the often precancerous, hyperplastic epithelial proliferations of human leukoplakia, the term "plaque" was adopted as an adequate designation to most simply identify these potentially precancerous growths. Leukoplakia has a Greek derivation and is defined as a white plate, plaque, or patch.

It is not uncommon for a portion of a conjunctival tumor to be a typical plaque and the remainder to be a papilloma. A few of these might actually be two tumors which have grown together but most are considered to represent a solitary growth which has mixed potentialities to develop into both a plaque and papilloma. In our tables, these mixed tumors were always classified as papillomas. Ash's observation [1,2] with regard to the primary sites of the epithelial conjunctival tumors was also consistent with our study. He observed

Fig. 6—Bilateral squamous cell carcinoma of the eye in an old cow. The eyelids were not involved (case D 2263).

that the conjunctival epithelium exposed through the palpebral fissure frequently becomes metaplastic and that this transformed epithelium is the important point of origin of the various kinds of epithelial tumors. Most of the plaques and papillomas in our series were about equally divided between the medial and lateral limbus and would be included in the area exposed when the eyelids were open. Russell and Loquvam [21] and Wynne and Russell [25] in preliminary reports on their studies of bovine ocular squamous cell tumors also adopted the use of the term plaque to indicate approximately the same type of lesion as we will describe in this article. No other investigators in this country have consistently used the term and few have made more than a brief mention of these benign or precancerous lesions.

Earlier, it was mentioned that plaques appeared on the skin of the eyelid and on the palpebral conjunctiva near its junction with the skin. In this survey, they were always found associated with squamous cell carcinoma when present on the eyelid. Sometimes, the carcinomas were still small and multiple benign tumors, including papillomas, made up the tumefied areas on either side of and often including the mucocutaneous junction (fig. 9). Although these plaques and papillomas were found only associated with eyes containing carcinomas and were, therefore, not included in the diagnoses listed in the tables, we believe from their appearance and reports of observations on afflicted herds [12, 13] that they are the predecessors of many of the carcinomas of the eyelid and palpebral conjunctiva. Apparently, epithelial tumors in these anatomic locations in cattle either undergo malignant transformation at an earlier date or are ignored by ranchers until they become carcinomatous. At least 59 malignant tumors of this type (tables 1 and 2) were examined in our survey.



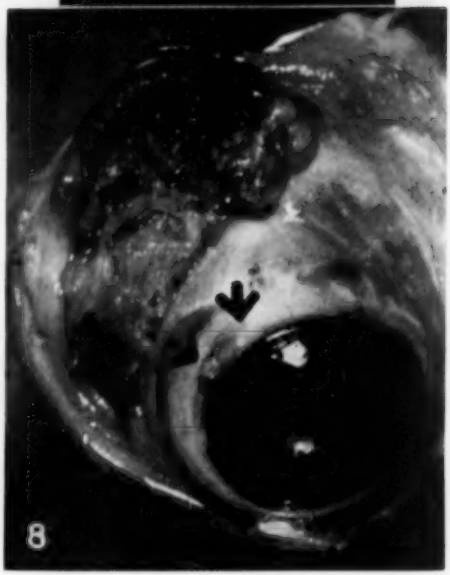


Fig. 7—An ulcerating squamous cell carcinoma (20 by 18 by 4 mm.) of the skin of the midsection of the lower eyelid and adjacent border of conjunctiva in an old Hereford cow. A squamous cell carcinoma of the eyeball from this same eye is shown in figures 35 and 37 (case 131808).

Fig. 8—A large ulcerating squamous cell carcinoma (4 by 2 by 1 cm.) almost completely replacing the membrana nictitans of an 8-year-old cow. Arrows outline two borders of a much smaller papillary lesion (7 by 3 by 1 mm.) on the limbus, which histologically (fig. 19 and 20) was regarded as an early squamous cell carcinoma (case D 2191).

There was often a pronounced hyperkeratosis of the tumefied epithelium of the papillary growths and this was often represented by cutaneous horn formation. Wynne and Russell's[51] use of the term "keratoma" to indicate this condition seems superfluous and actually confusing. Likewise, their coining of "boggy lids" to indicate a type of precancerous acanthosis of the skin of the eyelid does not seem to be justified until criteria are advanced to separate these lesions from early plaques arising in this same area.

One severe type of bovine conjunctivitis (fig. 11), which has been confused at necropsy with plaques and papillomas, has been considered similar to vernal catarrh of man and may represent a chronic allergic reaction.

The hyperplasia and sometimes great inflammatory enlargement (2 or 3 times) with formation of distinct lymphoid follicles of the membrana nictitans is the inflammatory lesion which is most difficult for us to differentiate from tumors on gross examination. Even microscopically, caution must be exercised in examining these nictitating membranes as a small tumor may be associated with a marked inflammatory reaction. This inflammatory lesion may occur alone or in association

Fig. 9—Arrows point to an area of squamous cell carcinoma of the eyelid at the lateral canthus in an old Hereford cow. Figures 22 and 23 are different magnifications of a histological section of this area which prompted the diagnosis of carcinoma.

The large papillary tumor on the lower eyelid was histologically typical of an early squamous cell carcinoma while the smaller growth above it on the upper eyelid was diagnosed as a papilloma.

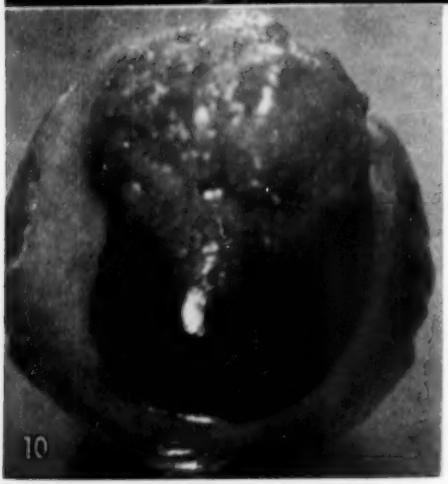


Fig. 10—Squamous cell carcinoma which replaced portions of the cornea and bulbar conjunctiva in same eye as in figure 9 (case 131788).

TABLE 1—The Location and Diagnosis of 613 Bovine Ocular Tumors

Location (lesion limited to anatomic area listed below)	Epidermal plaque	Epidermal papilloma	Early squamous cell carcinoma	Squamous cell carcinoma
Cornea	3	2	—	12
Bulbar conjunctiva	2	—	—	1
Bulbar conjunctiva and cornea	69	35	29	291
Eyeball and palpebral conjunctiva	—	—	—	19
Eyeball, palpebral conjunctiva, and skin of eyelid	—	—	—	12
Membrana nictitans	—	—	—	9
Palpebral conjunctiva and membrana nictitans	—	—	—	24
Palpebral conjunctiva (membrana nictitans not involved)	—	1	1	23
Palpebral conjunctiva and skin of eyelid	—	—	—	58
Skin of eyelid	—	—	—	1
Orbital region growth*	—	—	—	16
No external ocular tumor but a parotid lymph node metastasis†	—	—	—	5
Totals	74	38	30	471

* No recognizable remnants of eyeball and eyelids in 14 of the carcinomas. † Found with an apparently noninvolved, operated, or healed enucleated eye.

with a neoplastic condition or an advanced keratitis.

In making the diagnoses listed in tables 1 and 2, it was necessary to set up diagnostic criteria for each type of tumor. In the histopathology of most diseases, one can select clear-cut examples of closely related conditions such as a plaque and papilloma but cases almost intermediate in morphology will be encountered. The excrescency of the benign growth and the invasiveness of those tumors designated as early carcinomas seemed to be the most controversial points and the ones that had to be accurately interpreted for a correct diagnosis.

After a review of the tumors diagnosed as benign (many existed in the same eye with a carcinoma), it became apparent that the classic papillomas exhibited an abnormal outgrowth or projection to a marked degree and usually only a slight tendency toward invasion of subconjunctival tissues or the substantia propria of the cornea. These are the tumors which owners often tell of pinching off with their fingernails. However, this might leave a potentially malignant basal area where a malignant tumor would eventually develop. In addition, it is not generally appreciated that it is sometimes impossible to separate the papillomas from the early or frank papillary carcinomas by their appearance in the living animal and this makes such removal of papillomas even more hazardous.

The thicker plaques which almost projected enough to be papillomas appeared to be the most potentially malignant of the

TABLE 2—Observations on the Behavior of 471 Squamous Cell Carcinomas of the Eye in Cattle

Location (lesion limited to anatomic area listed below)	Squamous cell carcinoma	Visible ulceration at necropsy	Penetration into anterior chamber of eyeball	Parotid metastases	Parotid and other metastases‡
Cornea	12	1	—	—	—
Bulbar conjunctiva	1	—	—	—	—
Bulbar conjunctiva and cornea	291	61	47	1	—
Eyeball and palpebral conjunctiva	19	14	12	—	—
Eyeball, palpebral conjunctiva, and skin of eyelid	12	12	8	1	—
Membrana nictitans	9	5	—	—	—
Palpebral conjunctiva and membrana nictitans	24	20	—	—	—
Palpebral conjunctiva (membrana nictitans not involved)	23	14	—	—	—
Palpebral conjunctiva and skin of eyelid	58	56	—	13	1
Skin of eyelid	1	—	—	—	—
Orbital region growth*	16	16	—	2	2
No external tumor but a parotid lymph node metastasis†	5	—	—	5	1
Totals	471	199	67	22	4

* No recognizable remnant of eyeball and eyelids in 14 of the carcinomas. † Found with an apparently noninvolved, operated, or healed, enucleated eye. ‡ Parotid metastases were found in each metastatic carcinoma.

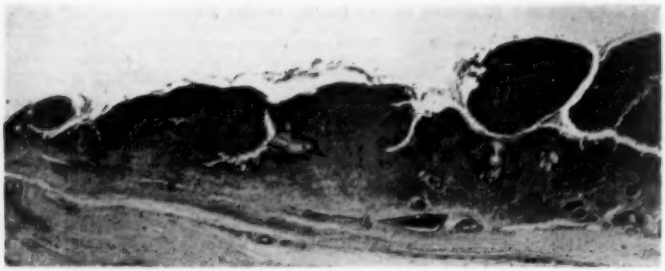


Fig. 11—A papillary keratitis which covered a 20- by 4-mm. area on the limbus of the eyeball of a 6-year-old Hereford cow. This lesion was erroneously diagnosed at necropsy as a papilloma (case D 2225). Hematoxylin-eosin stain. x 20.

benign group and, on gross examination, were indistinguishable from small invasive carcinomas. On the other hand, the transparent or partially opaque plaques were consistently represented only by a thickening of epidermalized epithelium and were obviously benign tumors. Complications with a secondary keratitis or conjunctivitis produced changes in appearance in many benign plaques and often made them difficult to distinguish from early carcinomas at necropsy and sometimes in microscopic examinations.

Histologically, the study of benign epithelial eye tumors is concerned with the changes in the four prominent layers of the epidermis: (1) basal cell layer (stratum germinativum); (2) prickle cell layer (stratum spinosum, rete mucosum, rete

Fig. 12—A portion of an ulcerating squamous cell carcinoma of the membrana nictitans and conjunctiva in an old Hereford cow is shown on the right. The growth on the lower segment of the limbus of the eyeball in the upper left was diagnosed as a plaque (fig. 16) and the tumor on the upper segment as a papilloma. The corresponding, incised parotid lymph node on the lower left contains an advanced metastasis (fig. 13). A plaque (not shown) was also found on the limbus of the opposite eye (case D 2270).

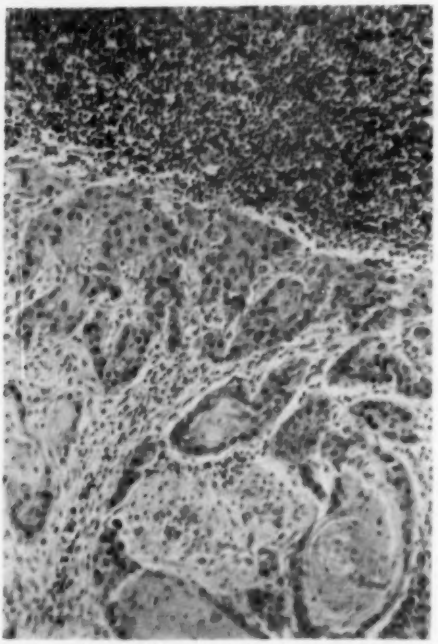


Fig. 13—A parotid metastasis of a squamous cell carcinoma of the eyelid. The appearance of this lymph node at necropsy is shown in figure 12 (case D 2270). Hematoxylin-eosin stain. x 100.

malpighii); (3) granular layer (stratum granulosum); and (4) cornified layer (stratum corneum). The distinguishing histological feature of a plaque is the hyperplasia or proliferation of the prickle cell layer (Ash descriptively called it "epidermidalization" of epithelium) in the absence of other distinctive changes in the growth. A thickening of two or three to ten or 15 times the normal height of the prickle cell layer was commonly seen.

There was no difficulty in justifying the diagnosis of a benign growth as a papilloma when there was a folding or ingrowth of epithelium to form inner connective tissue cores or even stalks. However, some benign growths, which had moderate proliferation of the prickle cell layer and marked hyperkeratosis or parakeratosis of the stratum corneum, posed a problem as to whether they should be called a plaque or papilloma. Grossly, these tumors were invariably considered as papillomas as they usually had multiple, rounded surface projections or a raised ragged exterior and actually were distinct outgrowths from the eye. Therefore, it was decided that the excrescency of the stratum corneum of these benign growths might be the deciding criterion of diagnosis. All four layers of the epidermis do proliferate in varying degrees in most of these localized tumors although the comparatively minimal enlargement in thickness of the basal and granular strata simplifies the classification to a consideration of the other two layers. In reviewing the papillomas with either well-defined basal stalks of attachment, projecting spines, or papillary protuberances with distinct connective cores, hyperkeratosis or parakeratosis was also found consistently.

Using the simplified histological system of classification listed later in this section,

Fig. 14—An epidermal plaque of the cornea and bulbar conjunctiva showing the characteristic thickening of the prickle cell layer. The appearance of this lesion at necropsy is illustrated in figure 1 (case D 2202). Hematoxylin-eosin stain. x 10.

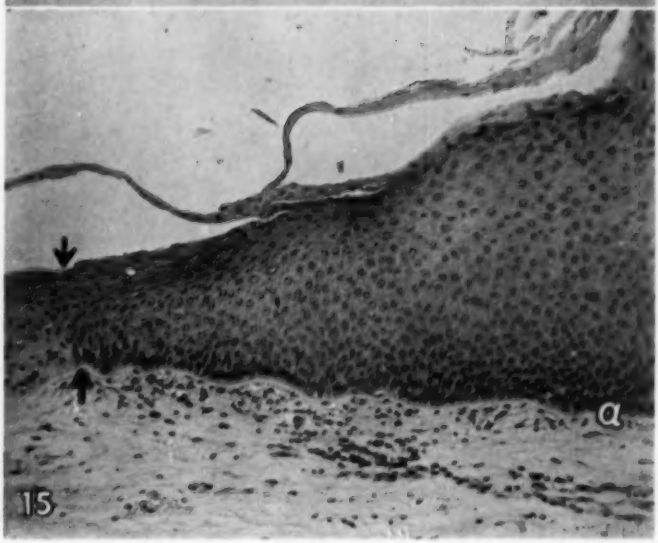


Fig. 15—A higher magnification of the left side of the lesion shown in figure 14 at the junction of the plaque and adjacent cornea (arrows). Note the proliferating prickle cell layer and the unusually well-defined Bowman's membrane (a) of the cornea (case D 2202). Hematoxylin-eosin stain. x 125.

a diagnosis of papilloma or plaque could best be correlated with the gross appearance of the lesions. This classification is the basis of diagnoses in this study. As expected, the greatest difficulty encountered on gross examination was in distinguishing between these benign lesions and the early or unequivocal carcinomas. It is doubtful, in this decade of science, if any diagnostic method except histological section can ever be used to accurately determine the lack of, or degree of, malignancy of the more superficial bovine eye tumors. A histological examination is, therefore, a necessary adjunct to the complete or partial surgical removal and evaluation of any epithelial eye tumor. Diagnoses of "carcinoma *in situ*" were not made, as it is our belief that many of the otherwise benign lesions would have at least one such focus if multiple histological sections were prepared. No one has yet developed means of determining whether these potential zones of malignancy have actually become carcinomatous. Carcinomatous changes apparently develop rapidly in the epithelial bovine eye tumors (a few months to more than a year), so until evidence is presented to refute our concept, the diagnosis of malignancy is based on neoplastic invasiveness of the tumors into the substantia propria. Metastasis was never observed until there was extensive invasion of the substantia propria.

Neoplastic invasiveness can be difficult to diagnose since there is no true basement membrane to the epithelium of the conjunctiva and skin. Also, the Bowman's membrane of the cornea is a thin, structureless layer (a homogenous tissue lacking fibers or cell nuclei) which has a tendency to disappear in inflammatory or proliferative conditions. However, lesions in which errors would be made, at best, would be only a small percentage of the total and would be of more academic than clinical interest.

The differential description of the four types of epidermal eye tumors used in classifying the neoplasms included in this paper are:

Epidermal Plaque.—A thickening of epithelium which is at least two times the normal and is a result of proliferation of the prickle cell layers so that they exceed the average height of the remainder of the tumefied epithelium (fig. 1, 14, and 15 represent a classic example of 1 type of lesion). The growths do not have a papillary surface although occasional inward

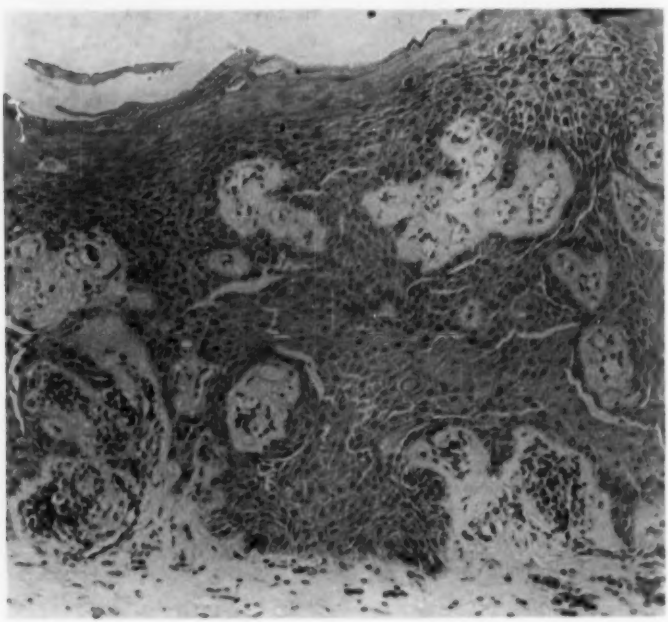


Fig. 16—An epithelial plaque in which there is an extensive ingrowth of the tumor. The general outline of this plaque on the cornea and bulbar conjunctiva can be seen in figure 12 (case D 2270). Hematoxylin-eosin stain. x 100.

growths of the tumor may cause islands of connective tissue resembling the connective tissue cores of a papilloma to be included in histological sections (fig. 16). Although Bowman's membrane of the cornea may appear thin and even disappear where there are corneal plaques, there is always an acceptable basal line to the tumefied epithelium of either the cornea, conjunctiva, or skin. Dyskeratotic changes such as hyperchromatism may occur in the thickened epithelium but usually are limited to individual cells. Formation of anaplastic nests of epithelial cells within the growths is rare. Subepithelial inflammatory reactions may be marked and there may be a pseudoepitheliomatous hyperplasia with great enlargement of rete pegs.

Epidermal Papilloma.—Included are all benign, papillary, epithelial outgrowths which have stalks, spines, or rounded protuberances represented by a central connective tissue core covered to a variable depth by a tumefied epithelium. In addition, benign tumors having a marked hyperkeratosis or parakeratosis of the stratum corneum, so that at least one projection of this cornified epithelium equals or exceeds the average height of the remainder of tumefied epithelium, are also designated as papillomas. Again, the Bowman's membrane of the cornea may appear thin and even disappear in places where there are corneal papillomas, but there is always an acceptable basal line to the tumefied epithelium of either the cornea, conjunctiva, or skin. Dyskeratotic changes such as hyperchromatism may occur in the epithelium and are more common than in plaques. Small, intradermal nests of anaplastic cells (carcinomas *in situ*), including such cellular phenomena as increased numbers of mitotic figures, enlargement of nuclei and nucleoli to form giant or sometimes double nuclei, great variation in size and shape of individual cells and small, isolated cornified bodies (epithelial pearls), may appear singly in the tumors, be found in several areas, or be absent. Subepithelial inflammatory reactions may be marked and there may be a pseudoepitheliomatous hyperplasia with great enlargement of rete pegs.

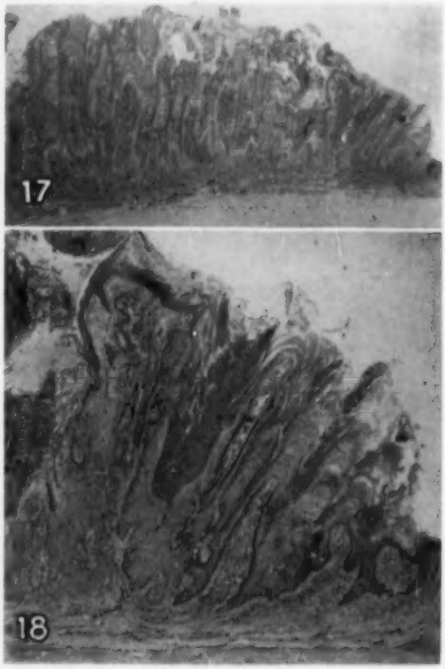


Fig. 17—An epidermal papilloma showing the deep indentations of the cornified layers of the neoplastic epithelium around central connective tissue cores. The appearance of this lesion at necropsy is illustrated in figure 3 (case D 2289). Hematoxylin-eosin stain. x 7.

Fig. 18—A higher magnification of the right side of the lesion shown in figure 17 (case D 2289). Hematoxylin-eosin stain. x 20.

Early Squamous Cell Carcinoma.—Nests of tumor cells at the base of an epithelial eye tumor appear to break through the basal cell layer of the tumefied epithelium and invade the subcutaneous tissues (fig. 8, 19, 20, and 21). If the tumor is on the cornea, the underlying Bowman's membrane is no longer intact or may have completely disappeared. On section, the nests usually appear as rounded subconjunctival

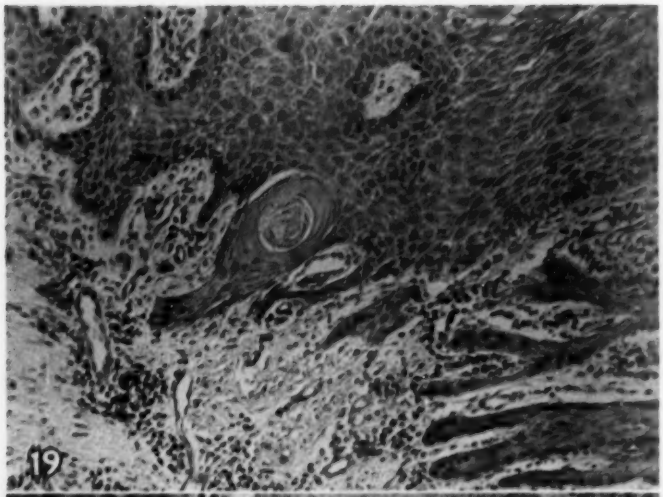


Fig. 19—An early squamous cell carcinoma arising at the junction of the cornea and bulbar conjunctiva of the eyeball whose appearance at necropsy was shown in figure 8. There are no longer distinct basal cell layers in the area which includes the epithelial pearl in the center of the illustration. Pseudoepitheliomatous hyperplasia shown at the right was prominent in the epithelium adjacent to the tumor (case D 2191). Hematoxylin-eosin stain. x 125.

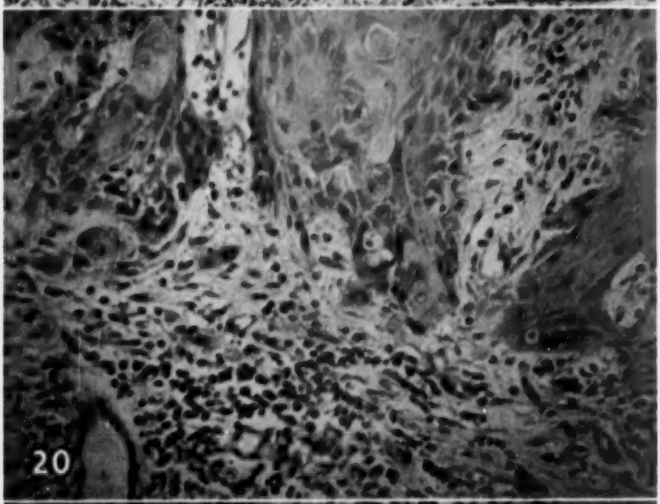


Fig. 20—A more anaplastic disruption of the basal cell layers of the tumefied epithelium of the early squamous cell carcinoma shown in figure 19 (case D 2191). Hematoxylin-eosin stain. x 170.

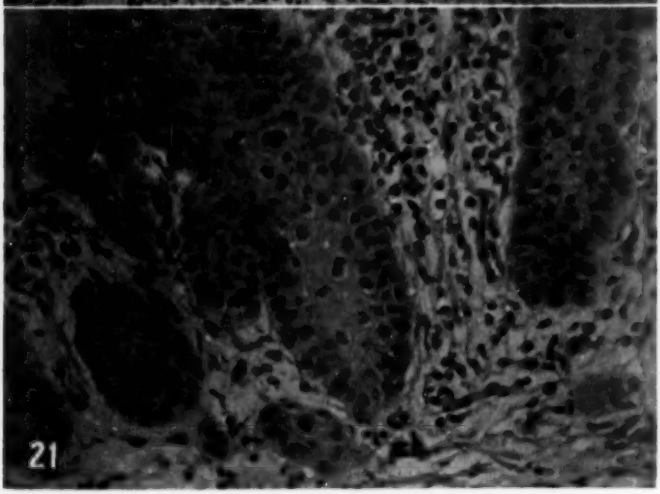


Fig. 21—The small, rounded groups of cells at the bottom of the figure were undoubtedly continuous with the branching ingrowth of the early squamous cell carcinoma before sectioning (an intact budlike projection of the epithelium can be seen in approximate center of illustration. This budding and disruption of the basal cell layers of the epithelium probably precedes but is often associated with deeper nests of carcinoma cells (case 131796). Hematoxylin-eosin stain. x 225.

groups of rather anaplastic cells and often have a central cornified center (sometimes advanced enough to be a classic "epithelial pearl"). The tumor, except for the carcinomatous changes in its basal area, may resemble a plaque or papilloma. However, as a group and compared with papillomas, there is a much greater tendency for the early carcinomas to form intradermal nests of anaplastic cells (carcinoma *in situ* described under epidermal papillomas). In addition, it is not uncommon for large portions of the basal cell layer of the tumefied epithelium to be replaced by these nests of anaplastic cells. Most of the invasive carcinomatous groups of cells still maintain intercellular bridges (prickles) particularly when there is cornification of their centers.

Occasional spindling of small groups of neoplastic cells is seen. In these areas, the cytoplasmic bridges are absent and the

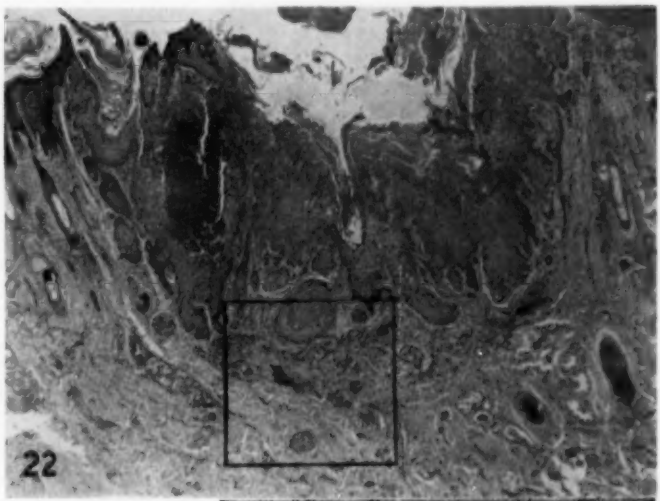


Fig. 22—A histological section of the squamous cell carcinoma of the eyelid indicated by arrows in figure 9. An area of invasive carcinoma is included within the blocked area and is shown at higher magnification in figure 23 (case 131788). Hematoxylin-eosin stain. x 35.

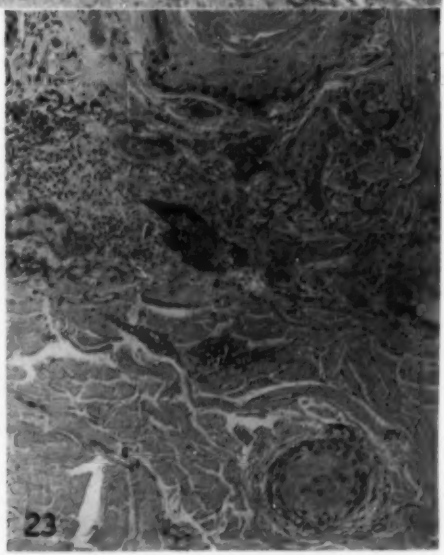


Fig. 23—A higher magnification of the area shown by a block in figure 22.

There is no longer an acceptable basal line to the tumefied epithelium and nests of tumor cells have invaded deep into the substantia propria of the conjunctiva (case 131788). Hematoxylin-eosin stain. x 80.

cells may be difficult to differentiate from those of a reticulum cell sarcoma or from epithelioid inflammatory cells invariably present as a component of a secondary tissue reaction. There may be marked pseudoepitheliomatous hyperplasia with greatly enlarged or elongated rete pegs in basal portions of the growths adjacent to the invasive areas. Usually, the rete pegs have slender and tapering projections in contrast to the rounded invasive neoplastic masses of cells which most characteristically distinguish an early carcinoma. The hyperplastic rete pegs may have a distinctive basal cell perimeter which can often be recognized even if the plane of the section results in a rete peg appearing as a single or several isolated groups of cells (fig. 26) in addition to the portion continuous with the epithelium. Subepithelial inflammatory reactions are prominent and include infiltrations of lymphocytes, plasma cells, fibroblasts and epithelioid cells, and increased vascularization and fibrosis of the substantia propria of the cornea and conjunctiva. Although the substantia propria of the cornea is normally almost devoid of blood vessels and lymphatics, such vessels do increase rapidly in number in the subepithelial tissues in apparent direct relation to the degree of irritation produced by the growing tumor.

Squamous Cell Carcinoma.—While the tumors classed as early squamous cell carcinomas were distinguished by what was considered to be one or more initial carcinomatous invasive ingrowths into the substantia propria, the full-fledged carcinomas include all eye tumors which have secondary or more advanced outgrowths from the invaded areas. There may be deep invasion through the substantia propria of the cornea to Descemet's membrane; lateral invasion under intact corneal, conjunctival, or cutaneous epithelium; or a reversed invasion through the tumefied epithelium (probably responsible for many of the ulcerating, necrotic surfaces). In the more differentiated carcinomas, multiple epithelial pearls may be found and it

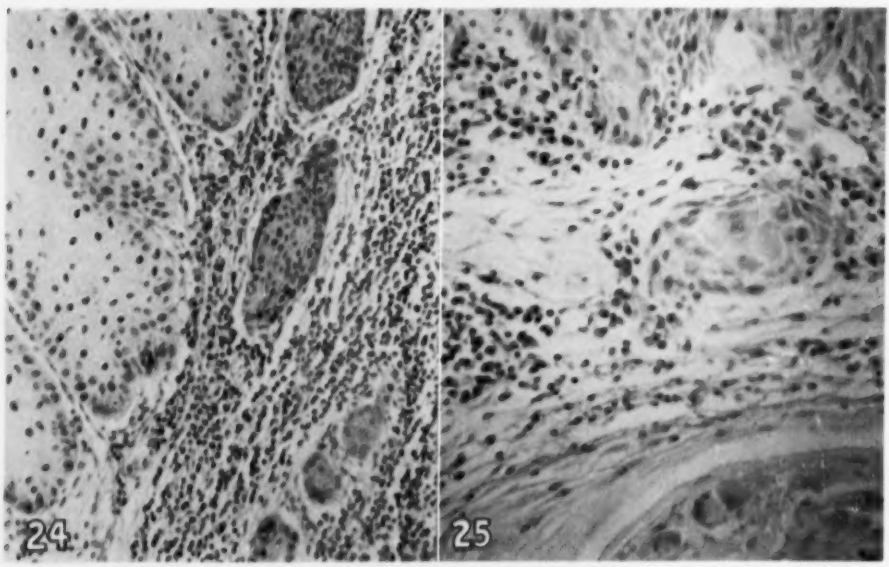


Fig. 24—Nests of tumor cells in this ocular squamous cell carcinoma in an old Hereford bull can be seen to be more anaplastic and invasive as they become separated from the still recognizable basal cell layers of the tumefied epithelium (case D 2313). Hematoxylin-eosin stain. x 140.

Fig. 25—A squamous cell carcinoma which covered only a 5- by 5-mm. area on the cornea of a 5-year-old Hereford cow. Numerous budlike nests of tumor such as the one shown in the right center were seen as well as deeper areas of obvious carcinoma (lower right) (case D 2304). Hematoxylin-eosin stain. x 210.

TABLE 3—Month of Collection and Evaluation of Ocular Lesions Encountered in 548 Cattle

Month	Unilateral neoplastic involvement	Bilateral neoplastic involvement	Keratitis	Corneal dermoid
January	23	3	2	0
February	47	6	1	0
March	33	13	1	0
April	17	3	0	0
May	7	0	2	0
June	8	0	1	0
July	10	2	0	0
August	25	12	1	1
September	26	0	1	0
October	38	0	0	1
November	169	9	1	2
December	77	4	2	0
Totals	480	52	12	4

is not unusual for individual pearls to reach a size large enough to replace one half of the normal depth of the cornea. Anaplasia and other malignant changes are much more advanced than those seen in carcinoma *in situ*. Sometimes, groups of epithelial tumor cells appear either fusiform or in extremely cellular arrangements in which cytoplasmic boundaries may be indistinct. Mitotic activity and formation of giant, double, or lobed nuclei usually increase in crude proportion to the malignancy of the tumor. A few tumors show definite neoplastic glandular formation in parts of their growths and an origin in or near glands associated with the eye is postulated. Scattered areas of necrosis are common in the larger tumors. In the more malignant tumors, invasions of veins or lymphatics may be demonstrable as well as metastasis to lymph nodes or body organs.

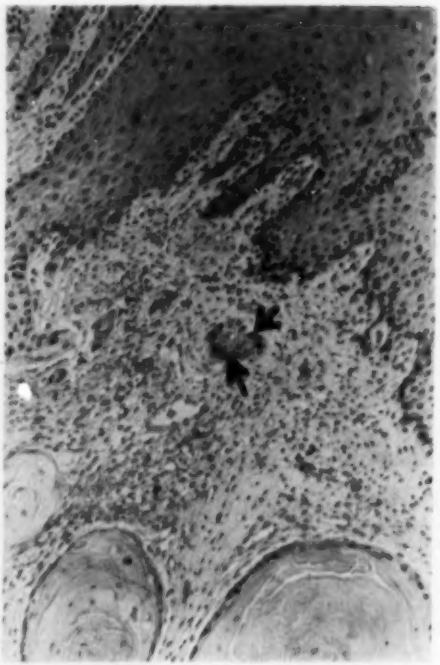


Fig. 26—An ocular carcinoma in an old Hereford cow which shows a cut section of an obvious, morphologically identifiable rete peg (arrows), three small budlike projections of tumefied epithelium to the left of the section of the rete peg, and an obvious carcinoma at the bottom (case 131679). Hematoxylin-eosin stain. x 110.

INTERPRETATIONS

The 613 bovine, squamous cell ocular tumors, shown in table 1, actually were found in 532 animals (table 3). Part of the discrepancy in numbers is explained in table 3 where it is indicated that 52 cattle had lesions in both eyes. In 29 other instances, two diagnoses were listed for a single eye. Two diagnoses were considered necessary when two independent tumors

Fig. 27—An almost complete section of a squamous cell carcinoma which was present at the limbus of the eyeball of a 5-year-old Hereford cow. One of the smallest carcinomas, it covered an area of only 5 by 2 mm. (case D 2297). Hematoxylin-eosin stain. x 28.

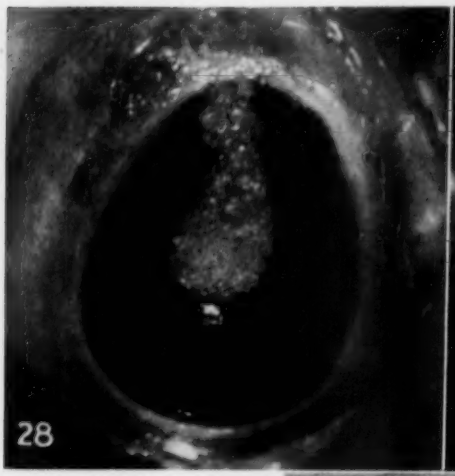
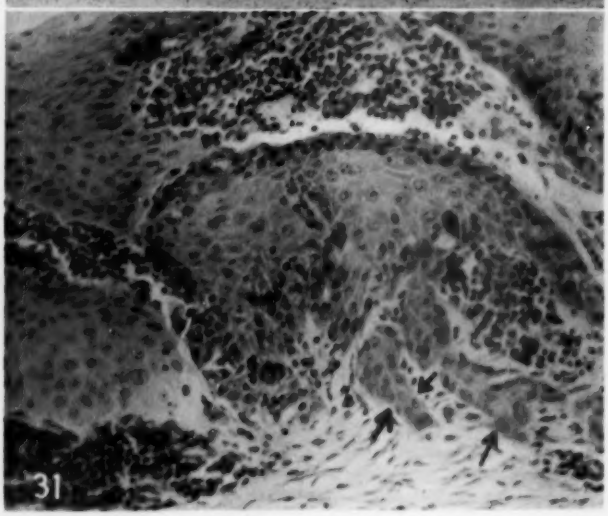


Fig. 28—A squamous cell carcinoma of the eye in a 9-year-old Hereford bull. Histologically, the cornea was invaded (fig. 30 and 31) only near the limbus. Other areas in the tumor appeared benign (case D 2237).

Fig. 29—A rather benign-appearings quamous cell carcinoma with a crescent-shaped pattern of growth on the cornea and bulbar conjunctiva and deep lateral invasion (fig. 36) of the cornea (case D 2190).

Fig. 30—A squamous cell carcinoma (see fig. 28), showing in the blocked area the point of invasive invagination and carcinomatous change. A higher magnification of this area is seen in figure 31 (case D 2237). Hematoxylin-eosin stain. x 45.

Fig. 31—Higher magnification of the invaginated epithelium shown in figure 30. Note the intact epithelium at the left of the invagination and how nests of tumor cells are invading the substantia propria (arrows) from the base of the area of neoplastic transformation (case D 2237). Hematoxylin-eosin stain. x 200.



with the same diagnosis were found in different anatomic locations in the same eye (arbitrary locations chosen as best suited to represent the most accurate and comprehensible analysis of our recorded data are given in tables 1 and 2). In compiling the data in the tables, only the diagnosis, or diagnoses, which represented the most

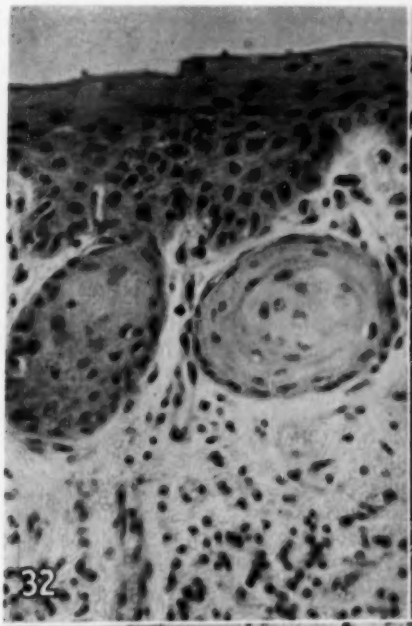


Fig. 32—Section taken near the border of a squamous cell carcinoma of the eyelid in a 9-year-old Hereford cow, showing two ball-like masses of neoplastic cells which are typical of those seen near the border of some squamous cell carcinomas of the eyelids. Note the point of attachment to the epithelium in the one at the left (case 130721). Hematoxylin-eosin stain. x 270.

Fig. 33—A squamous cell carcinoma of the membrana nictitans in a 6-year-old Hereford cow. Note the tremendous thickening of the tumefied epithelium and the normal epithelium at the right side in an area not yet completely distorted by the tumor (case 131428). Hematoxylin-eosin stain. x 30.

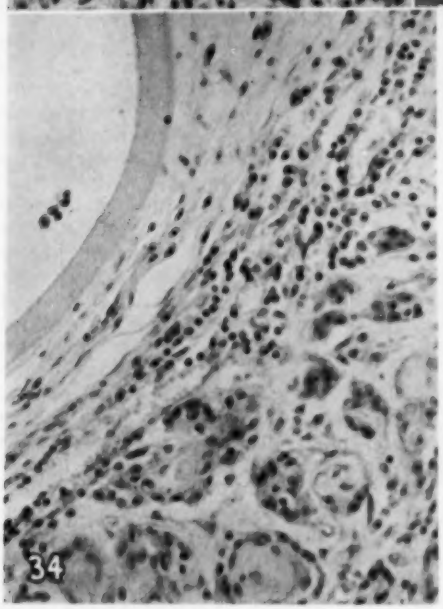


Fig. 34—Squamous cell carcinomas invading the cornea seldom penetrated completely to Descemet's membrane (upper left). In this carcinoma, found in a 6-year-old Hereford cow, a narrow fibrous and vascular connective tissue zone separated the tumor from the membrane (case D 2293). Hematoxylin-eosin stain. x 260.

TABLE 4—Estimated * Ages of 453 Cattle with Squamous Ocular Tumors

Years old												
Yearling heifer	2	3	4	5	6	7	8	9	10	11	12	Old animal†
1	4	4	4	9	35	47	68	16	14	4	1	247

* To nearest year. † Veterinary inspector did not make any additional or specific estimate of age.

advanced neoplastic change was listed. Therefore, a diagnosis of early squamous cell carcinoma was not included in our data if a squamous cell carcinoma was present in the same eye. In addition to including two squamous cell carcinomas in two different sites, a plaque and papilloma (plaques and papillomas are considered as having an equal precancerous potential) or either two papillomas or plaques found with no associated carcinoma were listed as two diagnoses unless they were identical diagnoses in the same anatomic site. Eyes in which there were three or more carcinomas or eyes with no carcinomas and three or more benign tumors in different anatomic sites were not found.

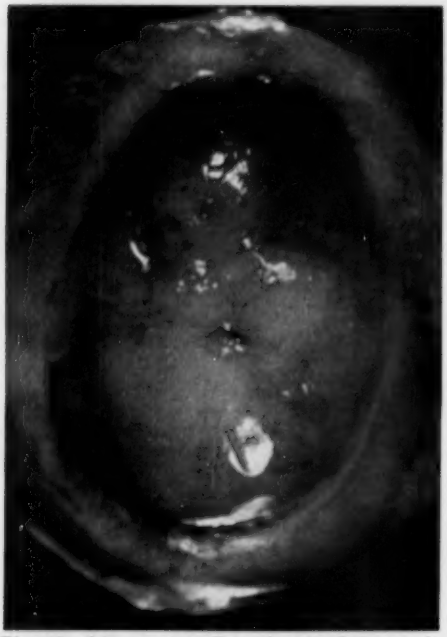


Fig. 35—A squamous cell carcinoma of the eye in an old Hereford cow which has extended extensively under the still-intact, corneal epithelium. The point of origin of the tumor could not be determined, although it may have been one of several, small, raised growths at the limbus. Figure 37 represents a histological section through the cornea of this eye and figure 7 is an associated eyelid lesion (case 131808).

Although the most advanced neoplastic lesion, or lesions, in each eye was included in our data, no attempt was made to evaluate the more benign tumors, one or more of which were found on 109 of these same eyes. Three illustrations (fig. 12, 13, and 16) represent the same case, a carcinoma of the eyelid with parotid metastases in which a plaque and papilloma of the eyeball were not recorded in our tables because they did not represent the most malignant lesions.

In the groups of plaques, papillomas, and early squamous cell carcinomas, there were 140 tumors of the eyeball as compared to two of the eyelids (table 1). When an evaluation of these squamous cell carcinomas was made, 304 of the 471 were confined to the eyeball, 115 were limited to the eyelids and membrana nictitans, and 31 had both eyelid and eyeball involvement. In the remaining 21 carcinomas, the tumor was present in the orbital region and no remnant of eyeball could be found except in two instances when squamous cell tumor deposits were found at the scleral perimeter of a structurally intact but atrophic eyeball. The atrophic corneas of both eyes were tumefied only at their margins and there were no extensions of the carcinomas to the conjunctiva of the eyelids. Extensive metastases were present in the corresponding parotid lymph nodes of both eyes. It is postulated that an incomplete removal of a corneal or conjunctival scleral tumor might produce this kind of lesion.

The data from table 1 would support the commonly expressed but poorly documented impression that tumors on the eyelids grow more rapidly and would be more apt to appear in abattoirs as advanced cases. Some allowance would have to be made for the difficulty in detecting small tumors arising in the membrana nictitans and the conjunctiva of the eyelids. However, in reference to the important group

TABLE 5—The Disposition of Cattle and Sheep Affected with Epithelioma at Denver's Federally Inspected Abattoirs During the Calendar Years 1952 and 1953

	Condemned on				Head condemned, remainder of carcass passed without restriction		Total No. of animals affected with epithelioma	
	Antemortem inspection		Postmorten inspection					
Species	1952	1953	1952	1953	1952	1953	1952	1953
Cattle	42	21	68	81	1,845	2,977	1,955	3,079
Sheep	—	—	1	—	—	—	1	—

— = no animals affected.

of usually multiple and, in early stages, benign tumors of the mucocutaneous junction of conjunctiva and skin, recognition of a probably neoplastic condition should be made as readily as in the instances where tumors involve the eyeball. It would be logical to expect that more of this kind of eyelid growths had been subjected to surgery or other treatment because of their objectionable appearance. There were indications that many if not all of the 21 orbital growths already discussed, as not directly associated with an eye lesion, represented the results of an unsuccessful surgical attempt to remove a primary ocular carcinoma.

Among the 532 cattle with ocular tumors, the breed of 385 was recorded and 378 were considered to be of predominant Hereford ancestry. The others were: Hol-

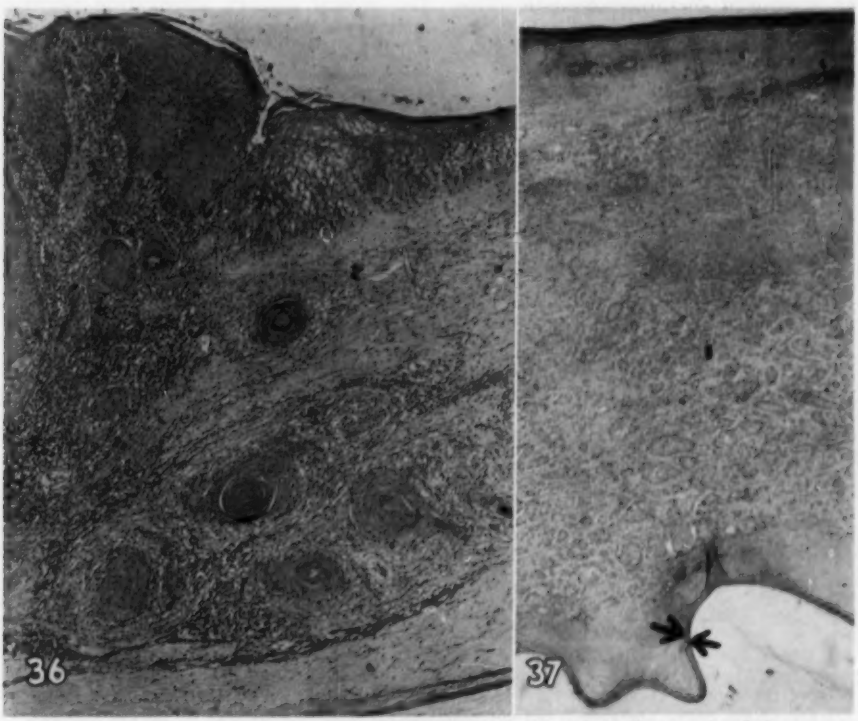


Fig. 36—Lateral invasion of a squamous cell carcinoma in the substantia propria of the cornea beneath nontumefied corneal epithelium. The appearance of this tumor at necropsy is shown in figure 29. Note the epithelial pearls in many of the deep nests of the carcinoma (case D 2190). Hematoxylin-eosin stain. x 45.

Fig. 37—A squamous cell carcinoma invading laterally under corneal epithelium. The tumor is confined to the cornea by a still-intact Descemet's membrane (arrows). The appearance of this eye at necropsy is shown in figure 35 (case 131808). Hematoxylin-eosin stain. x 20.

stein-Friesian, 4; Guernsey, 2; and Shorthorn, 1. An effort was made to recover ocular tumors from Aberdeen Angus and the increasingly popular crossbred Hereford and Aberdeen Angus cattle but none was found during the year. Carcinomatous eyes from such animals have never been submitted to this laboratory.

The estimated ages of 453 of the 532 cattle included in this study are listed (table 4). Considering that cattle slaughtered in the Denver abattoirs are predominantly under 6 years of age, many of our cases would appear to represent older breeding animals which had been culled by the stockmen. They included 3 Hereford steers, 2, 3, and 7 years old. Tumors were submitted from 9 Hereford and 1 mixed-breed bulls, 3 of which were listed as 8 years; 1 each as 6, 9, and 10 years of age; and 4 as old animals.

The gross and photomicrographic illustrations were selected primarily to show our interpretation of the basis for a diagnosis of early squamous cell carcinoma and squamous cell carcinoma. Any growth not having the invasiveness of an early carcinoma would be classed as either a plaque or papilloma. Observations made on 74 plaques, 38 papillomas, and 30 early squamous cell carcinomas (table 1) show the average arithmetical diameters of plaques as 7.5 mm., papillomas as 13.9 mm., and early squamous cell carcinomas as 13.5 mm. Plaques projected an average of 1.4 mm. from the free surface of the eye, as compared to 6.7 mm. for papillomas and 4.3 mm. for early squamous cell carcinomas. These figures indicate that papillomas are not only the largest but also are the most excrescent of the three types of tumors. The comparatively small size of several of the early carcinomas and unequivocal carcinomas encountered in this survey was amazing. The dimensions listed for several tumors, illustrating pertinent diagnostic points in classification, are typical for the size at which a small but appre-

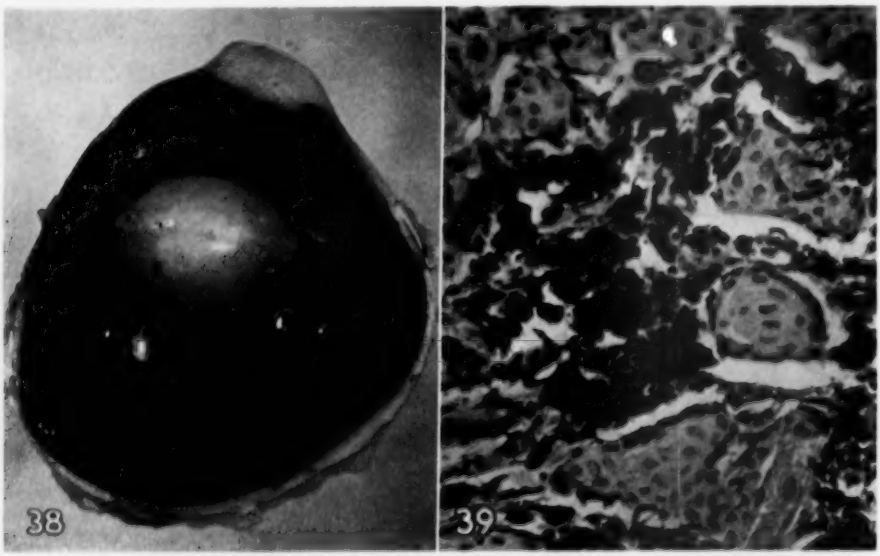


Fig. 38—A horizontal meridional section of a tumefied eyeball in a 10-year-old Hereford cow. Apparently, the tumor was at first confined to the cornea and bulbar conjunctiva but later invaded the anterior chamber of the eye and produced a proliferation of the pigment of the iris and ciliary body. The growth did not penetrate the posterior chamber of the eye although it forced the lens (center) and other attached structures to be displaced into the cavity of that chamber. The microscopic appearance of the tumefied iris is shown in figure 39 (case 131543).

Fig. 39—Section of tumefied iris (see fig. 38) showing proliferating but not neoplastic pigment cells and deposits of their pigment intermixed with irregular masses of cells of a squamous cell carcinoma of the eye (case 131543). Hematoxylin-eosin stain. x 260.

ciable percentage may undergo malignant transformation (fig. 8, 9, 25, 27, and 28).

Several of the more common types of invasiveness of an early squamous cell carcinoma are shown in photomicrographs (fig. 19 to 21) and the further invasiveness of an unequivocal carcinoma is also illustrated (fig. 22 to 27, 30, 31, 33, 34, 36, 37, and 39 to 41). There may be indecision in the differentiation of an early squamous cell carcinoma from a benign lesion but, under our classification, there should be no hesitation in making a diagnosis of carcinoma.

In figures 30 and 31, we have an example of what Willis [29] designated as invasive invagination of the epidermis in the early stages of transformation of a squamous cell carcinoma. There is an invasive growth of tumor cells at the base of the invaginated epithelium to establish the diagnosis of squamous cell carcinoma. A phenomenon closely related to, or a part of, invagination which may be helpful in diagnosis is shown (fig. 32). At the borders of eyelid tumors, we frequently see these ball-like masses of neoplastic squamous cells. They sometimes have cornified centers (epithelial pearls) and usually are still attached by a narrow neck to the epithelium or appear free in the substantia propria (probably a section of a tubular invagination of a tumefied epithelium). In all instances, frank carcinomas have been associated with these tumefied masses in other sections of the lesions, although seeing them only with a benign tumor, one may hesitate to classify them as squamous cell carcinomas. These balls are probably closely related to the budlike projections associated with early squamous cell carcinomas (fig. 21).

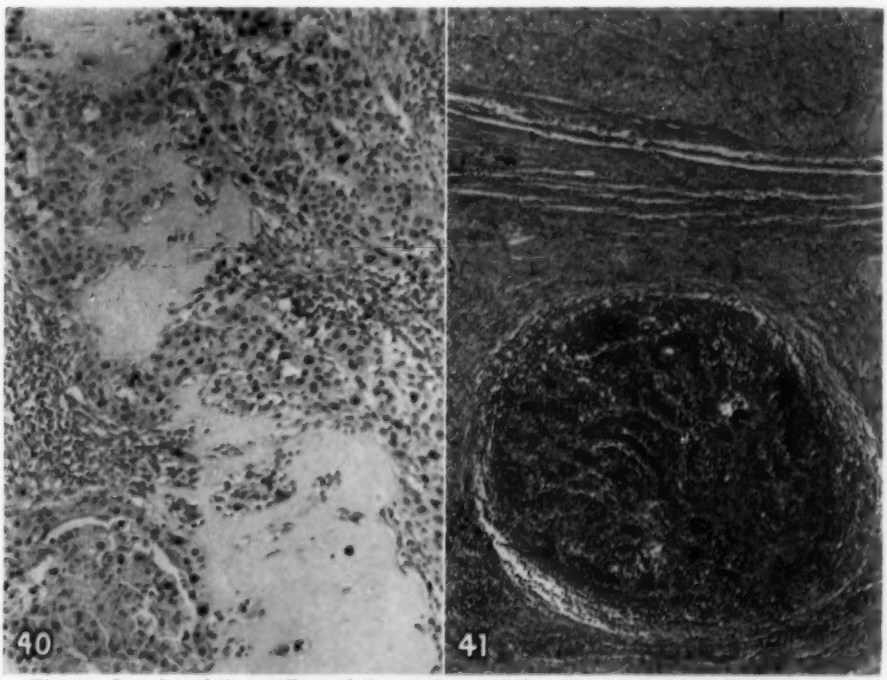


Fig. 40—Invasion of the cartilage of the membrana nictitans by a squamous cell carcinoma of the eyelid in an 8-year-old Hereford cow (case 131370). Hematoxylin-eosin stain. x 105.

Fig. 41—A typical cordlike extension of a squamous cell carcinoma of the eyelid into tissues behind the eyeball. This tumor in an old Hereford cow was growing along the perimeter of the optic nerve which is seen in the upper portion of the illustration (case 131749). Hematoxylin-eosin stain. x 30.

Once secondary invasion from a primary lesion of a squamous cell carcinoma has taken place, several interesting general patterns of growth are seen. On the eyeball, there may be direct and deep invasion by carcinomas of the substantia propria of the cornea until growths nearly penetrate to Descemet's membrane. This elastic membrane, in contrast to Bowman's membrane of the cornea, appears to be quite resistant to neoplastic penetration; we have never observed its penetration by a carcinoma. Usually the tumors approach until only a narrow fibrous and vascular connective tissue zone separates them from the membrane (fig. 34), then they grow laterally rather than invade through the membrane (fig. 36 and 37).

On gross examination of some tumors which involve the eyeball, a grayish, opaque cornea with a smooth, glistening surface can be seen (fig. 35). If there are any associated, projecting tumor masses, they may cover a large portion or small segments of the cornea (fig. 10 and 29). In other eyes, there are no visible external growths. In eyes with an opaque cornea related to an ocular squamous cell carcinoma, it was found that a tumefied and thickened cornea was responsible for the opacity (fig. 35 and 37). In the eyes with corneal carcinomas which had no apparent external point of origin, there may have originally been a papilloma. If its external surface was removed, its base could easily have been overgrown by proliferating corneal epithelium.

A more deep and lateral spread of the eyeball tumors, which originate at the limbus, sometimes finds tumors growing around the border of Descemet's membrane and into the scleral meshwork and the iris angle of the eye. They sometimes continue as a growth into the ciliary muscle and ciliary processes, but a more spectacular route is to invade the iris. If the iris becomes progressively tumefied, it may attain a remarkable size (fig. 38) and adhesions will usually form to anchor it to both Descemet's membrane and the lens. Cells of the pigmented epithelial layer and of other pigmented areas in the iris and ciliary body undoubtedly multiply and also become scattered among the tumor cells; a black, fibrous tumor mass is consistently encountered on section of these eyes. At first, a complicating melanoma of the ciliary body, a tumor not rare in man, was considered as a possible necropsy diagnosis in addition to the obvious carcinoma of the cornea and sclera which was always seen with it. However, on histological section (fig. 39), there was a consistent picture of nests of neoplastic squamous epithelial cells similar to those seen in the primary tumor on the conjunctiva or cornea intermixed with masses of pigment. In the stroma of these iridal growths, poorly defined cells containing cytoplasmic pigment granules are frequently seen and these may be hyperplastic pigment-producing cells. Ash[2] mentions iris invasion as being of rare occurrence in man. This is undoubtedly true as it is seen in bovine eyes which have obviously been tumefied for many months longer than would be

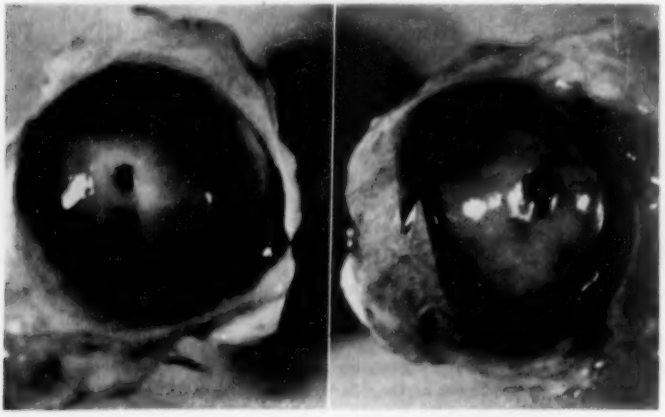


Fig. 42—A bilateral subacute keratitis in an 8-month-old Holstein-Friesian calf. A fistulous tract in the center of each cornea extended into the anterior chamber of each eyeball (see fig. 45) (case 130803).

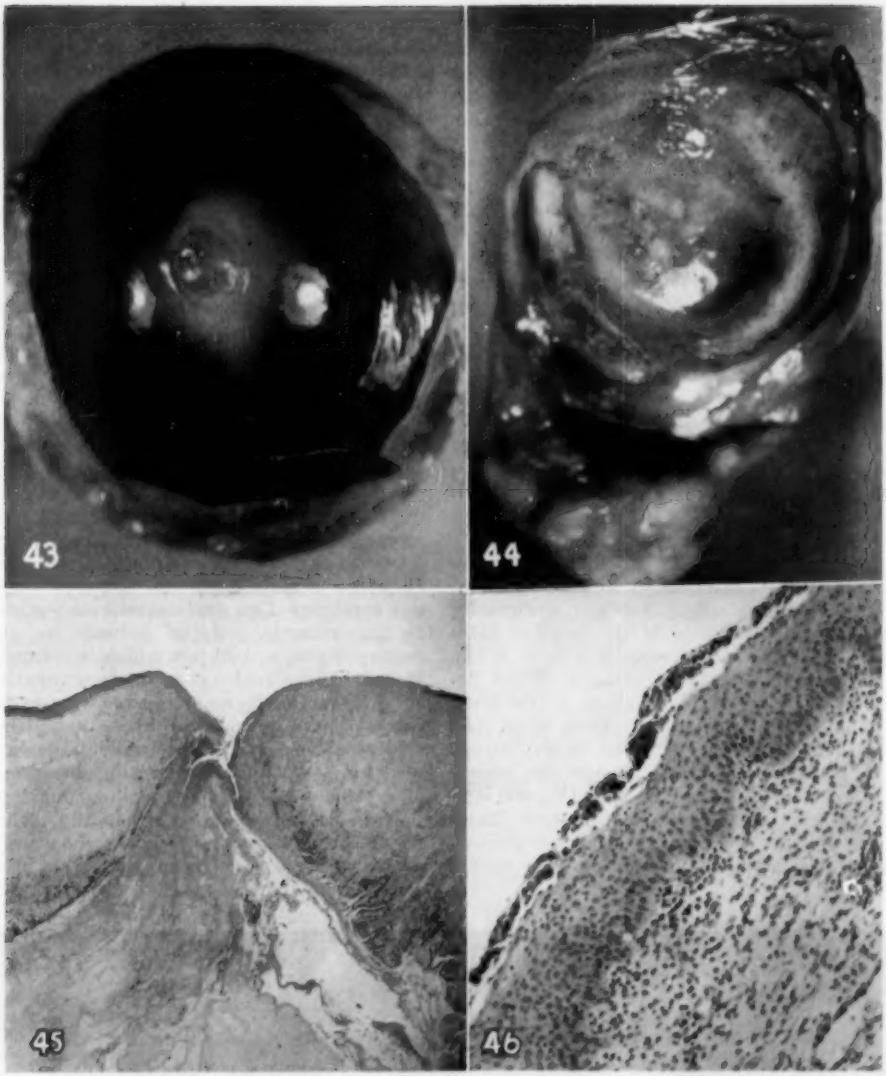


Fig. 43—A unilateral, subacute keratitis in a 1-year-old Aberdeen Angus steer. The keratitis did not produce a fistula into the anterior chamber of the eye (case 130820).

Fig. 44—A chronic unilateral keratitis in a 6-year-old Hereford cow. A photomicrograph of a section of cornea of this eye is shown in figure 46 (case 130492).

Fig. 45—A subacute, ulcerative keratitis which has extended through the cornea as a fistula into the anterior chamber of the eyeball, also showing a slight proliferation of iridal pigments along the borders of the fistula. There is often a proliferation of iridal pigments in these fistulas (case 130803). Hematoxylin-eosin stain. x 15.

Fig. 46—A chronic keratitis. The appearance of this eye at necropsy is shown in figure 44. Note the inflammatory membrane covering the intact corneal epithelium (case 130492). Hematoxylin-eosin stain. x 110.

tolerated by human patients. The lesion should not be confused with melanomas.

The limbus-iris route of invasion was probably followed in many, if not all, of the 67 carcinomas (table 2) which had penetrated into the anterior chamber. Our criterion of penetration into the anterior chamber was for the tumor to form adhesions with, or to contact, the posterior or the endothelial lined surface of Descemet's membrane. Tumors invading by way of the iris are actually confined to the anterior chamber and prevented from re-entering the substantia propria of the cornea by an intact Descemet's membrane. Evidence of invasion into the anterior chamber in 67 of 335 cases of carcinoma of the eyeball (table 2) indicates that this is a common sequela of advanced cases of the disease.

The great invasiveness of a rapidly growing and malignant tumor of the eyelid and membrana nictitans can be shown in many ways and the invasion of cartilage (fig. 40) is an excellent example. Primary skin or conjunctival tumors invading the orbital tissues often seem to grow in cord-like extensions (fig. 41) and eventually may invade the bone of the orbit if fatal metastasis has not already occurred. While the carcinomas may grow around and behind the eyeball to produce marked atrophy of that structure, there is no evidence in our study of direct invasion into the posterior chamber through the sclera. In some instances, there may be extension of the carcinoma into the posterior chamber of the eye by progressive growth around a partially or completely displaced lens.

It has been mentioned that squamous cell carcinomas frequently have a reversed invasion which carried the proliferating areas back to the surface of the growth. We believe that this reversal is more common in conjunctival and skin tumors and is indirectly responsible for the frequently encountered surface ulcers and necrosis. Other factors contributing to the necrosis and ulceration would include bulkiness, secondary inflammatory reactions, and circulatory phenomena which may result from an uncontrolled new growth of tissue.

The data, compiled under the heading of visible ulceration at necropsy (table 2), are of particular interest from a meat inspection standpoint. These ulcers were visible as demarcated areas in which the surface of the tumor was replaced by black-to-yellow, soft, slimy, or watery material (fig. 7, 8, and 12). They were usually associated with a foul odor and necrosis which might be found throughout the tumors. In such cases, the heads are appropriately condemned immediately after they are removed (if the carcasses were not condemned on antemortem inspection) as the necrotic material adheres to, and contaminates, any object which it contacts. Deeper inflammatory and degenerative conditions, such as secondary keratitis, conjunctivitis, lymphadenitis, cellulitis, and sometimes an appreciable secondary necrosis, were found in varying degrees in all of the malignant eye tumors but were not considered under this heading. They would have importance in the disposition of heads. We were interested strictly in the heads which would unequivocally be

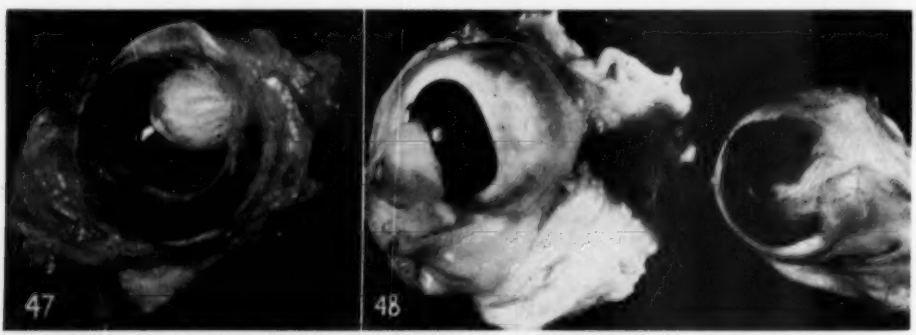


Fig. 47—A unilateral corneal dermoid in a 2-year-old Hereford steer (case 131408).

Fig. 48—Bilateral corneal dermoids in an old Hereford cow (case 131562).

a source of contamination to the meat packer.

Ulcers were found only in tumors considered to be ocular carcinomas. The relative proportion of ulcerated carcinomas in varying anatomic sites can be found by comparing columns 1 and 2 of table 2. Of 471 carcinomas, 199 (roughly 2 out of 5) had an ulcerated surface. While slightly more than one of five (62 of 304) carcinomas confined to the eyeball were ulcerated, more than four of five (137 of 167) involving extra-eyeball ocular structures had visible and unquestionable ulcers. No disposition except condemnation of the heads can be made when an ocular carcinoma with an ulcerated surface is present.

DISCUSSION

Surgical procedures followed by veterinarians in the treatment of early or moderately advanced epidermoid tumors of the bovine eye, as a rule, are much more drastic than actually required to completely remove the tumefied areas. This may be in part due to a lack of understanding of the behavior of these neoplasms. In the West, most stockmen feel that, economically, only a single visit by a veterinarian to treat possibly several animals in a beef cattle herd which are afflicted with eye tumors can be afforded. However, it is deplorable when enucleation becomes the only method of treatment considered worthwhile by a practitioner.

Frank,[12] in 1943, outlined the basic surgical procedures which should be employed in the treatment of these tumefied eyes and they are still applicable. He mentioned the desirability of removing small tumors of the cornea, membrana nictitans, or eyelid with a sharp scapel. Such surgical specimens should be submitted to a histopathology laboratory to see that the base and outer borders include all of the tissues invaded by the neoplastic process. Failure of the surgeon to effect a complete removal would justify a second and more drastic operation. How easily a tumefied basal area might be left behind in a papillary carcinoma unless a generous excision is employed is illustrated (fig. 4 and 5). *Beta* radiation, radioactive cobalt, and radon have been used by a few veterinarians[6, 26, 28] in selected cases of bovine, epidermoid, ocular tumors with apparent good results. When facilities exist, such

Fig. 49—A malignant lymphoma of the bulbar conjunctiva of the eyeball in a 6-year-old Holstein-Friesian cow is shown in the upper portion of the picture. Ordinarily, this lesion is easily differentiated at necropsy from a squamous cell carcinoma (case D 3108).

treatment may be more satisfactory and desirable especially during the periods of the year when surgical wounds would have to be protected from flying insects.

Although the epidermoid eye tumors are comparatively more rare in man than in the bovine population, the same basic problems exist in their treatment. Ash,[2] in 1950, reported that many eye enucleations were done in man in the treatment of epithelial eye tumors finally diagnosed as benign when simple excisions would have resulted in complete recovery, thus saving the eyesight. Ash warned of the dangers of recurrence unless the surgeon either before or after the operation made a careful slit-lamp examination of the conjunctival epithelium to assure eradication of all altered epithelium.

Recognition of epidermoid tumors and their differentiation from other non-neoplastic conditions is often possible when cattle are examined at antemortem inspection. Doubtful lesions can always be examined carefully at postmortem inspection. It has previously been mentioned that the hyperplastic and inflammatory enlargement of the membrana nictitans and the vernal catarrh of the eyelid conjunctiva might resemble a plaque or papilloma.

Acute and subacute lesions of infectious bovine keratoconjunctivitis (pinkeye) should not be confused with ocular carcinomas. Figure 42 is an illustration of a subacute, bilateral keratitis which might have been an ulcerative form of the disease. This lesion was also distinctive microscopically (fig. 45). The chronic forms of the disease in individual animals with no available herd history are the most difficult to differentiate from epidermal tumors. A keratitis which might be a chronic form of pinkeye or some other progressive keratitis is shown (fig. 44 and 46). No evidence of neoplasia was found in the eye shown in figure 44 but a unilateral chronic keratitis such as is shown in the figure warrants a careful examination.

Some cases of traumatic keratitis have suspicious areas which have to be studied histologically to eliminate a diagnosis of a co-existing primary carcinoma. It is impossible to determine the etiological factors of most cases of subacute keratitis at necropsy in meat inspection. Traumatism may often be responsible for a unilateral keratitis such as is shown (fig. 43).

There were four examples of conjunctival "dermoid" in our survey which might have been confused with an epidermal tumor. They were found at the limbus of the eyeball as a unilateral involvement (fig. 47) and as a bilateral involvement (fig. 48). Runnells [20] described the embryonic development of dermoids from skin and accessory skin structures and regarded them as neoplastic-like disturbances of development. He mentioned that an unusual location for dermoid in Hereford cattle was in the eyelid and on the cornea. All of our cases were found in Herefords. Ash [1,2] and Ash and Wilder [3,4] described these same embryonic dermoids in man. Schmidt,[22] in 1941, observed dermoids as abnormalities in 2 calves born to Jersey cows on a low carotene diet. Schmidt's idea that the dermoids were a manifestation of a vitamin A deficiency was not proved by his experiments.

Eye involvement in cases of malignant lymphoma is not uncommon, but generally there is no basis for confusing these lesions, either at clinical examination or necropsy, with those of carcinoma of the eye. Growth of a malignant lymphoma in the bulbar conjunctiva produced lesions in one eye in our tumor survey (fig. 49) which had a slight resemblance to a conjunctival carcinoma. Lesions found in other anatomic locations in this case, and in a second case with involvement of the membrana nictitans, made a necropsy diagnosis of malignant lymphoma almost a certainty before histological examinations were conducted.

The finding of squamous cell carcinoma at the corneal-bulbar conjunctiva junction in over 50 per cent of our cases (table 1) is in general agreement with observations of Steiner and Bengston [23] and of Russell and Loquvam [21] on similar groups of tumors observed in specimens collected from federally inspected abattoirs. We saw fewer tumors (2%) limited to the membrana nictitans than did Russell and Loquvam (17%) and we wonder if they did not include tumors involving the skin of the eyelid in their 22 per cent found on the mucous membrane of the eyelid. They [21] made no mention of the skin-conjunctival tumors which represented over 12 per cent of our ocular carcinomas. The sharp differentiation of epithelium at the conjunctival-skin junction of the eyelid is a more obvious embryological junction of epithelial surfaces than the limbus. If these two last-mentioned areas of the eye are the zones where tumors are more apt to occur, when they are subjected to irritation, it is not difficult to imagine at least an equal exposure of the eyelid to wind, dust, and sunlight as that which takes place to portions of the limbus exposed through the palpebral fissure.

There has been only meager or incomplete reference to the occurrence of multiple ocular squamous tumors in single bovine eyes and even less in regard to bilateral involvement. Frank [12] reported never seeing bilateral involvement. Guilbert *et al.*[13] observed multiple wartlike masses as unilateral or bilateral growths on the lower eyelid of cattle but never more than a unilateral development of a malignant tumor in these same eyes. In the section on interpretations, it was indicated that 52 of the 532 cattle had bilateral neoplastic involvement. It was also mentioned that 29 affected eyes had multiple tumors of equally important diagnostic significance in different anatomic locations and that 109 had additional, independent but more benign growths than those which were diagnosed

and recorded in table 1 as the most malignant tumor or tumors of the affected eye. These figures overlapped in regard to multiple lesions on single eyes. To summarize the data on multiple tumors, 148 (28%) of the 532 cattle with ocular squamous tumors either had additional, multiple, independent growths or a bilateral involvement.

The 4.7 per cent occurrence of parotid metastases in the present study was much lower than the 15 per cent of Russell and Loquvam.[21] It is not the purpose of this paper to go into the behavior and characteristics of metastatic ocular carcinomas. We did section many extremely hyperplastic and enlarged parotid lymph nodes and in no instances did we find metastases which were not evident at necropsy. Steiner and Bengston's observation [23] that this carcinoma metastasizes lymphogenously late in its development is supported by the present survey. The single case of parotid metastasis from a carcinoma of the eyeball (table 2) is an indication of the rarity of that phenomenon when only the eyeball is involved. However, there is no way of determining how many original eyeball carcinomas spread by infiltration to involve the eyelids and then metastasize by the lymphatics to the parotid lymph nodes.

Our criterion that an orbital region growth contain no recognizable remnant of eye (table 1 and 2) was an absence of any identifiable structural part including pigment. Pigment, if present, could be assumed to be derived from a remnant of the ciliary body, iris, choroid, or retina. We have found other remnants of eye in orbital masses in which initial histological sections had revealed only pigment and organized inflammatory tissues.

It was estimated that 82 per cent of the slaughtered cattle at the Denver abattoirs in recent years were of a predominant Hereford breeding, 3 per cent of other beef breeds (including an increasing yearly number of Aberdeen Angus), and about 15 per cent of dairy breeds. This was the consensus of Denver veterinary stockyard inspectors, veterinary meat inspectors, brand inspectors, and meat packers and their employees. Cattle going through the Denver stockyards as feeders or for general sale, particularly in the fall months, may well be over 95 per cent of the Hereford breed but this estimate is of no importance in evaluating the occurrence of cancer eye in slaughtered cattle.

The contention that November and December are the months when most cancer eye cattle are slaughtered is supported by the monthly breakdown for the collection (table 3). Cows considered to be poor wintering risks or near the end of their reproductive life are culled on many ranches in the late summer or fall. This close inspection of cattle generally results in the inclusion of many older cows with diseased eyes in the shipment of unprofitable breeding animals.

The age of the animals in relation to the month in which they were slaughtered was not listed (table 3) but, in tabulating the data, we did observe that more of the cows estimated to be in the 4- to 9-year-old group were slaughtered in the January to July period. Cattle from whom eyes were collected in September to January were not only older animals but usually had extensive lesions. The estimated average age of the affected cattle in our series would be at least 8 years (table 4) as compared to the average of 5 years reported by Russell and Loquvam.[21]

Our observation supports that of others in previously published papers that squamous cell carcinoma of the bovine eye is a tumor which usually metastasizes only after a long period of progressive growth. Those originating on the eyeball metastasize only in the most advanced stages. There is only meager recorded data on the follow-ups of cattle afflicted with eye tumors in respect to local recurrence of the growths after treatment. Correlated information on the clinical and microscopic appearance of the neoplasms, and the method and result of treatment, would be valuable data.

The probable inherited susceptibility of certain families of cattle to carcinoma of the eye and the questionable value of salvaging an afflicted animal for additional use as a member of a breeding herd are problems of importance to the breeder, geneticist, and veterinarian. No attempt was made to identify the herd origin of our cases so these problems will only be mentioned. Investigators are conducting long-term studies on individual herds and their preliminary reports indicate that there is a higher hereditary predisposition

to the development of bovine ocular carcinoma in some bloodlines.

SUMMARY

A total of 722 ocular squamous cell tumors, collected at necropsy from 532 cattle, were studied to determine their more distinctive patterns of growth and to establish a basis for their classification. They were found in a 12-month survey of bovine eye tumors in three Denver abattoirs. Of the 532 cattle, 52 had bilateral squamous cell tumors.

It was proposed that benign ocular squamous cell tumors be designated as epidermal plaques or papillomas and their malignant counterpart as early squamous cell carcinomas or squamous cell carcinomas.

A detailed study was made of 613 of the ocular tumors, using the most malignant neoplasm or group of neoplasms from each eye studied. The other 109 tumors were independent tumors of a more benign classification occurring as one or more additional growths on the affected eyes; they were not used as a source of data in the evaluation of the behavior of individual ocular tumors.

Of the 613 tumors, 471 were diagnosed as squamous cell carcinomas, 38 as early squamous cell carcinomas, 38 as epidermal papillomas, and 74 as epidermal plaques.

Of the 471 carcinomas, 22 had metastasized to the parotid lymph nodes and four of these had additional metastatic lesions.

There were 199 carcinomas which had ulcerated surfaces and would have been a source of contamination if the lesions had not been detected in the inspection procedures.

Of 335 carcinomas involving the eyeball, 67 had penetrated into the anterior chamber of that structure.

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Virulence of *Leptospira Pomona* in Hamsters and Cattle

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THE SYRIAN HAMSTER is being used extensively in the study of leptospirosis but there is little information in the literature on the virulence of *Leptospira pomona* in these animals. The objectives of the investigation described in this report were to study the effect of serial passage on the virulence of *L. pomona* in hamsters and to obtain some information on the effect of this hamster passage on the virulence of *L. pomona* for cattle.

LITERATURE REVIEW

Meyer *et al.*[6] observed that very young guinea pigs are susceptible to infection with *Leptospira canicola* and that the chief signs of the disease were a febrile reaction accompanied by loss of weight. They also added that death in guinea pigs was rarely seen, but continued serial passage in these animals tended to increase the relative number of deaths. Walch-Sorgdrager[13] pointed out that the disease in guinea pigs due to infection with *L. canicola* was much milder than that caused by *Leptospira icterohaemorrhagiae*. Packchanian[10] studied the susceptibility of 32 species and subspecies of rodents to virulent strains of *L. icterohaemorrhagiae*. He concluded that certain species of American deer mice, genus Peromyscus, were suitable as susceptible laboratory animals for experimental studies and for diagnosis of Weil's disease. Larson[5] stated that white mice *(Mus musculus)* were extremely susceptible to *L. icterohaemorrhagiae*.

Syrian hamsters were first investigated by Morton[9] for their susceptibility to leptospiras. Using a single strain of *L. canicola*, he found that hamsters survived the infection although the organisms could be detected in the blood. Larson[6] reported that hamsters *(Cricetus auratus)* are susceptible to infection with both *L. icterohaemorrhagiae* and *L. canicola* and are of value in isolation and identification of leptospiras found in man and animals. He also noticed that *L. icterohaemorrhagiae* caused jaundice, hemorrhage, and death in guinea pigs, mice, and hamsters within four to ten days. Larson added that *L. canicola* killed hamsters, while it caused only a febrile reaction with loss of weight in guinea pigs.

Randall and Cooper[11] concluded that the golden hamster is the animal of choice for isolation of leptospiras, especially *L. canicola*, since young guinea pigs and mice are resistant to infection and rats are entirely refractory. They stated that *L. canicola* and *L. icterohaemorrhagiae* were found to produce a fatal infection in hamsters in five to ten days. Van Der Hoeden[12] reported that guinea pigs, albino mice, hamsters, voles, and gerbiles were susceptible to a number of strains of leptospiras. He added that illness and death occurred occasionally with *L. icterohaemorrhagiae* and *L. pomona*.

MATERIALS AND METHODS

Hamsters used in this investigation were obtained from Lakeview Hamster Colony, New Field, N. J. A supply of healthy hamsters was kept in colony cages, while infected ones were kept in separate cages in another building. They were fed commercial rabbit pellets containing approximately 15 per cent crude protein, 2 per cent crude fat, and 18 per cent crude fiber, with no added antibiotics.

Cattle used in this experiment were from the experiment station herd and were maintained in separate pens in an isolation building. They were fed a ration of hay and ground grain containing approximately 15 per cent protein, with no added antibiotics. All were negative for leptospiral antibodies at the time of exposure.

The strain of *L. pomona* used for experimental infections was originally isolated from the urine of a naturally infected cow in August, 1953.* The organism was identified as *L. pomona* and was designated "Hardacre strain." The strain was maintained on Schüffner's medium with occasional passages in hamsters or guinea pigs. The strain of *L. pomona* utilized for serological tests had been previously obtained from the Army Medical Center and was designated (NIH). Four- to 7-day-old cultures were used in the agglutination-lysis test.

Schüffner's medium as modified by Verwoort[14] was used for the cultivation of leptospiras. About 0.1 to 0.2 ml. of blood, aseptically obtained by cardiac puncture in hamsters or from the jugular vein in cattle, was inoculated directly into each of two tubes of Schüffner's medium. These tubes were incubated at 30 C. and examined by dark field microscopy at weekly intervals to detect the presence of leptospiras. The culture was consid-

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* Obtained from Dr. E. H. Bohl, Department of Bacteriology, Ohio State University, Columbus.

ered negative when no organisms could be detected after four to five weeks.

Search for leptospirae in tissues of hamsters which died or were destroyed after infection was made on coverslip preparations of fresh tissue suspensions using dark field microscopy at a magnification of 150 or 600. The organisms were also demonstrated in tissue sections by the silver-impregnation method.[7]

Cattle were exposed to leptospiral infection with a suspension of known infected hamster tissue. One milliliter of blood and about 5.0 Gm. of liver from an infected hamster were ground with 5.0 ml. of Schüffner's medium in a tissue grinder. Five milliliters of the homogenate were injected intramuscularly in cattle and 0.1 ml. intraperitoneally into 1 or 2 hamsters. The inoculum was examined by dark field microscopy before injection to determine the presence of living leptospiras. Because of technical difficulties, an estimation of the number of leptospiras was not done. When two or more organisms were observed in each microscopic field (x 600), the inoculum was considered satisfactory.

RESULTS

A) The Effect of Serial Passage on the Virulence of Leptospira Pomona in Hamsters.—Direct serial passage in hamsters, about 3 to 5 weeks old, was started on March 22, 1955, by intraperitoneal inoculation of 0.1 ml. of a culture of *L. pomona* (Hardaere strain). Blood was collected by cardiac puncture from the hamsters four days following inoculation and part of it was inoculated into two tubes of Schüffner's medium and 0.1 to 0.2 ml. of the fresh blood was also injected intraperitoneally into 1 or more susceptible hamsters. This direct animal to animal passage was continued with the results indicated (table 1).

In the early passages, infected hamsters survived; however, signs of generalized icterus were observed on the ninth and tenth passages. The inoculated hamsters of the early passages recovered from the disease but, when they were destroyed as late as 84 days after exposure, the kidneys were grossly involved and leptospiras were demonstrated by dark field microscopy. At the sixteenth passage, both inoculated hamsters died four days after injection and the transfer was thereafter made on the third day. Mortality approximated 100 per cent and, as the passage progressed, most of the hamsters died by the third day. At the twenty-sixth passage, the hamsters died on the fifth or sixth day following exposure and this series was terminated at the twenty-eighth passage.

A second series of passages was initiated with a blood culture from a hamster which had been injected with urine from a cow experimentally infected with the Hardaere strain. The blood transfers were made on the fourth day up to the sixteenth passage and then on the third day after that to make this series comparable to the first. Since this reduced interval appeared to result in a loss of virulence of the organisms, the transfer was made on the fourth day after the twenty-first passage. Results of this experiment were comparable to those obtained in the first series except that the increase in virulence was not so rapid. At the twenty-sixth passage, the hamsters died on the tenth day and, as the passage progressed, the virulence of the organisms increased. The hamsters were dying by the third day at the thirty-first passage, at which time the transfer was made on the third day. The hamsters of the thirty-seventh passage died on the sixth and seventh days (table 2). Hemoglobinuria was present at the time of death in the forty-

TABLE 1—Changes in Virulence of Leptospira Pomona Following Serial Passages in Hamsters

No. of passage	No. of hamsters	Time of bleeding	Blood culture *	Infected organs †	No. recovered	No. died	Remarks
1st	2	4th day	+	.....	2	0	0.1 and 0.5 ml. of *L. pomona* culture injected.
7th	2	4th day	+	Liver, kidney.	....	....	Both used to expose bull 107.
16th	2	4th day	+	Lung, liver, kidney.	0	2	Both died on day 4.
17th	2	3rd day	+	Lung, liver, kidney.	0	2	Died on days 3 and 4.
20th	3	3rd day	+	Lung, liver, kidney.	0	3	All died on day 3.
23rd	4	3rd day	+	Lung, liver, kidney.	0	4	2 died on day 3, 1 on 4, other used for exposure.
25th	2	3rd day	+	Lung, liver, kidney.	0	3	1 died on day 3, other used for experiment.
26th	2	3rd day	+	Lung, liver, kidney.	0	2	1 died after bleeding, other on day 6.
27th	2	3rd day	+	Lung, liver, kidney.	0	2	Died on days 5 and 6.

* Isolation of leptospiras from blood culture. † Isolation of leptospiras from organs.

TABLE 2—Second Serial Passage of Leptospira Pomona in Hamsters

No. of passage	No. of hamsters	Time of bleeding	Blood culture	Infected organs	No. recovered	No. died	Remarks
1st	2	4th day	+	.....	2	0	0.1 and 0.5 ml. of *L. pomona* culture injected.
7th	2	4th day	+	Lung, liver, kidney.	....	....	Both used to expose 1201 and 695.
16th	2	3rd day	+	.....	2	0	.....
19th	4	3rd day	+	Kidney.	2	2	2 died on day 17.
21st	4	4th day	+	Kidney.	0	4	1 died on each of days 11, 13, 21, and 30.
27th	2	4th day	+	Kidney.	0	2	Died on days 7 and 10.
29th	2	4th day	+	Lung, liver, kidney.	0	2	Died on days 4 and 5.
30th	2	4th day	+	Lung, liver, kidney.	0	2	Both died on day 4.
31st	2	4th day	+	Lung, liver, kidney.	0	2	Died on days 3 and 4.
32nd	2	3rd day	+	Lung, liver, kidney.	0	2	Died on days 3 and 6.
37th	2	3rd day	+	Kidney.	0	2	Died on days 6 and 7.
39th	2	3rd day	+	Kidney.	0	1	1 died on day 6, other used for exposure.

fourth and fifty-fourth hamster passages and leptospiras were demonstrated in the urine of both animals by dark field microscopy.

The effect of freezing temperatures on leptospiras in infected tissues collected during the acute phase of the disease was determined. Hamsters of the twenty-fourth, twenty-seventh, and twenty-eighth passages of the first series were destroyed on the third day of infection when they were moribund. Liver tissue, cardiac blood, and a whole kidney were placed in separate pyrex tubes and were frozen in dry ice and 95 per cent alcohol, then stored at a temperature of about —60 C. Numerous leptospiras were demonstrated in all these tissues by dark field microscopy before freezing. The frozen blood was thawed 24 hours after freezing and it was noticed that most of the leptospiras were nonmotile. Two hamsters were inoculated with 0.5 ml. of each material eight months after freezing and no leptospiras were recovered from the blood culture and no mortality was recorded.

The minimum lethal dose of hamster tissue of the twenty-seventh passage of the first series was determined in young hamsters. One-tenth milliliter of the 10^{-3} dilution of ground liver tissue caused death in a hamster at eight days.

A second attempt was made to measure the infectivity and minimum lethal dose, using tissue of the fifty-first passage of the second series. Tenfold dilutions of liver homogenate were prepared in Schüffner's medium, including concentrations of 10^{-1} to 10^{-10}. Five-week-old hamsters were inoculated with 0.2 ml. of each dilution. The 10^{-9} dilution caused leptospiremia and death in 1 hamster (table 3).

B) The Effect of Hamster Passage on the Virulence of Leptospira for Cattle.—Jersey bull 107, 1 year old, was exposed to infection using hamster tissue of the seventh passage, first series. The animal died seven days following exposure. A de-

TABLE 3—Measuring Infectivity in Hamsters Using Leptospira Pomona, Fifty-First Passage

Dilution of tissue suspension	No. of injected hamsters	Dark field examination of inoculum *	Mortality †	Infectivity †	Remarks
Undiluted	2	+	2/2	2/2	Both died on day 4.
10^{-1}	2	+	2/2	2/2	Both died on day 4.
10^{-2}	3	+	3/3	3/3	2 died on day 8, 1 on day 13.
10^{-3}	3	+	3/3	3/3	2 died on day 11, 1 on day 18.
10^{-4}	3	+	3/3	3/3	Died on days 14, 23, and 34.
10^{-5}	2	Few +	1/2	2/2	1 died on day 16.
10^{-6}	1	—	1/1	1/1	Died on day 12.
10^{-7}	1	—	1/1	1/1	Died on day 12.
10^{-8}	1	—	1/1	1/1	Died on day 13.
10^{-9}	1	—	1/1	1/1	Died on day 12.
10^{-10}	1	—	0/1	0/1	Killed after 5 wk., kidneys negative.

* Demonstration of leptospiras in the inoculum by dark field microscopy. † Numerator indicates the number affected, denominator the number exposed.

tailed description of the signs and lesions is given in a separate report.[3] Two young bulls, 28 and 700, were inoculated with 5.0 ml. of hamster tissue of the twenty-third passage, first series. Two hamsters were given 0.1 ml. of the same tissue suspension and both died by the fourth day. Bull 28 was born two months after his dam was experimentally infected at seven months of gestation, but his serum was negative for leptospiral antibodies at the time of exposure when he was 5 months old. No signs of illness were recorded, except a slight rise of temperature on the first day following inoculation. Neither leptospiras nor their antibodies were demonstrated until 26 days after exposure, at which time there was a positive reaction in 1:10 and 1:20 serum dilutions. The animal remained essentially normal (table 4).

Jersey bull 700, 1 year old, showed no clinical signs of illness except a slight rise of temperature. He maintained a good appetite. Infection was present as evidenced by the isolation of leptospiras from the blood after three days and the appearance of antibodies after the fifth day. The maximum titer was 1:1,600 reached 16 days following exposure.

During the second serial passage in hamsters, cattle were also exposed to infection at the early and late hamster passage to compare the results obtained with those of the first series. A pregnant heifer, 1201, 2 years old, and bull 695, 1 year old, were exposed to infection using hamster tissue of the seventh passage. Two hamsters were also infected with 0.1 ml. of the same material.

The pregnant heifer aborted 20 days following exposure. The bull became extremely ill and, on the third day, developed a fever of three days' duration. The highest titer, 1:6,400, was observed 23 days following infection. Leptospiras were isolated from his blood on the first to fourth days and the organisms were recovered from his urine three weeks after infection. The bull recovered. When slaughtered, seven months later, the only gross lesions were in the kidneys which were pale with reddish brown spots about 2 mm. in diameter.

Holstein-Friesian bull 1161, 2 months old, was injected intramuscularly with 5.0 ml. of liver homogenate of the thirty-ninth hamster passage and, at the same time, a hamster was inoculated intraperitoneally with 0.1 ml. of the same infective agent used to expose the young bull. This hamster died four days following inoculation.

The calf showed a febrile response with temperatures reaching 104.0 to 106.8 F. This response was the only clinical manifestation of the disease. Leptospiras were isolated from the blood on the fourth day following infection. The antibody titer reached 1:6,400 at 26 days following exposure.

Pathological Findings.—In the acute phase of leptospirosis in hamsters, the lung showed gross hemorrhages only after the ninth serial passage and dark field microscopy revealed the presence of leptospiras. It was noticed that as the passage continued the lesions were more extensive. Microscopic examination of the lung sections stained with hematoxylin and eosin

TABLE 4—Clinical, Serological, and Bacteriological Observations on 5 Bulls and a Pregnant Heifer Following Inoculation with Leptospira Pomona Using Early and Late Hamster Passages

Animal No.	Clinical observations	Isolation of *L. pomona* from	Agglutination-lysis test titer
Early passage			
107	Febrile on days 3 and 4—maximum 105.4 F., anorexia, icterus, leukopenia, anemia, hemoglobinuria, died on day 7.	Blood after day 3, kidney after day 7.	1:10 after day 5, 1:160 after day 7.
1201	Febrile on days 4 to 6—maximum 105.8 F., anorexia after day 5, aborted on day 20.	Blood after days 1 to 3, urine after days 19, 21, 23, 25.	1:20 after day 6, 1:25,600 after day 9.
695	Febrile on days 3 to 6—maximum 105.7 F., anorexia.	Blood after days 1 to 4, urine at day 16.	1:40 after day 6, 1:6,400 at day 23.
Late passage			
28	Transient febrile response of 104.0 F. on day 1.	Negative.	1:10 after day 26.
700	Transient febrile response of 104.4 F. on day 1.	Blood at day 3.	1:20 after day 5, 1:1,600 at day 15.
1161	Febrile days 3 to 5—maximum of 106.8 F.	Blood at day 4.	1:1,600 at day 11.

revealed hemorrhages and leukocytic infiltration (lymphocytes and monocytes) and broken alveolar walls. Emphysema and consolidation were present as a result of hemorrhage. Leptospiras were demonstrated in lung sections stained with a silver-impregnation method.

The liver was markedly congested and leptospiras were demonstrated only during the leptospiremic phase of the disease, using dark field microscopy or hamster inoculation. Histological examination of the liver section revealed massive hemorrhages and lymphocytes were present around the large interlobular vessels. Necrobiosis of the liver cells was evident throughout the section.

The kidneys were grossly involved; they were highly congested with numerous petechial hemorrhages. Leptospiras were demonstrated by dark field examination, by hamster inoculation, or in tissue sections stained with the silver-impregnation method. Microscopic examination showed massive hemorrhage in the renal tubules and glomeruli. Bowman's capsule and glomerular tufts were destroyed in many places and hyaline degeneration was present in other renal tubules and glomeruli.

In the chronic phases of the disease, the lungs and liver were grossly normal and no organisms could be demonstrated. The only lesions regularly found in animals with chronic infections were those involving the kidneys. They were generally enlarged, pale, and pitted in appearance. Leptospiras were demonstrated in the kidneys, and they tended to clump together and become immobilized within a few minutes after opening the kidney. As a result, the process of dark field examination or animal inoculation with the kidneys was completed as quickly as possible to avoid this immobilization. Microscopic examination of kidney sections revealed an increase of interstitial connective tissue (fig. 1). Degeneration of epithelium of the renal tubules and broken tubule walls were found in some sections. The glomeruli were shrunken and some were edematous. Most renal tubules and Bowman's spaces contained some debris.

Discussion

Leptospira pomona, Hardacre strain, increased in virulence for hamsters as the serial passage progressed in these animals. In the early passages, the hamsters were readily infected but mortality was low. When they were killed as late as 84 days after infection, the kidneys were grossly involved and leptospiras were demonstrated by dark field microscopy. The kidneys were enlarged, pale, and pitted in appearance. This was due to the kidney damage resulting from extensive involvement of the parenchyma with interstitial nephritis following the localization of leptospiral organisms.

It became apparent that a change was taking place in the virulence of the organisms for hamsters at the sixteenth serial passage. Instead of the usual chronic cases with low mortality, the inoculated hamsters were dying within four days. The passage was continued until hamsters were dying on the third day. These peracute cases, resulting in 100 per cent mortality, occurred between the sixteenth and the

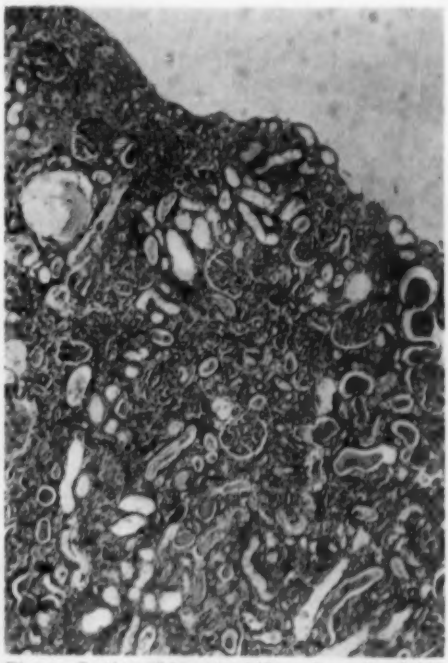

Fig. 1—Section of hamster kidney from the eighth passage, destroyed 69 days following infection with Leptospira pomona. Many renal tubules have been destroyed with a corresponding increase in interstitial tissue. The remaining tubules are dilated, some containing heme casts. Glomeruli in various stages of degeneration are shown. Hematoxylin and eosin stain; x 90.

twenty-fifth hamster passages. Apparently, the virulence of the organisms could not be increased beyond this point and, in fact, it seemed to decrease after the twenty-sixth passage since the hamsters died on the sixth and seventh days although mortality was still high.

It is possible that the blood transfer on the third day instead of the fourth day played a role in this delay of death. Probably the leptospiral multiplication reached its maximum on the fourth day, so the number of the organisms in the inoculum was much greater than that on the third day. This was supported by observations in the second serial passage and also in the experiment designed to measure the minimum lethal dose and infectivity of *L. pomona* in hamsters. The hamsters, which were given the undiluted tissue suspension and that diluted to 1:10, died on the fourth day while those given the dilutions of 1:100 to 1:1,000,000,000 died in eight to 34 days after the injection. The susceptibility of hamsters to the disease apparently depended to a certain extent on age since in some previous work 3- to 5-week-old hamsters died earlier than older ones.

Leptospira pomona caused a generalized infection in hamsters and the organisms were isolated during the leptospiremic phase of the disease from the blood, liver, lung, spleen, and kidneys. The circulating leptospiras may form an embolus in the blood stream as illustrated (fig. 2). If these emboli lodge in small vessels, infarction may occur with resulting hemorrhage and necrosis. Hemorrhages, more common in acute cases, may be produced in the lungs and can be considered to be the result of a localized toxic effect of the leptospiras as well as direct invasion of the tissues. When antibodies are formed, the organisms disappear from the blood stream and other tissues but tend to localize in the kidneys producing leptospiruria long after they have disappeared from the blood.

The strain was highly virulent in cattle only when the early hamster passage was used. The Hardacre strain was highly virulent for bulls 107 and 695 and cow 1201, all of which developed severe signs of the disease following infection using hamster tissue of the seventh serial passage. The signs and lesions of leptospirosis were most severe in bull 107 which died seven days following exposure to infection. Bull 695 showed similar clinical signs. He recovered but continued to eliminate leptospiras in his urine. It is entirely possible that bulls are important as carriers of the disease. If leptospiras remain with the residual urine in the urethra, these organisms may be transmitted during breeding. Therefore, carriers should be eliminated from a herd and attention should be directed toward avoiding the use of such animals for breeding purposes.

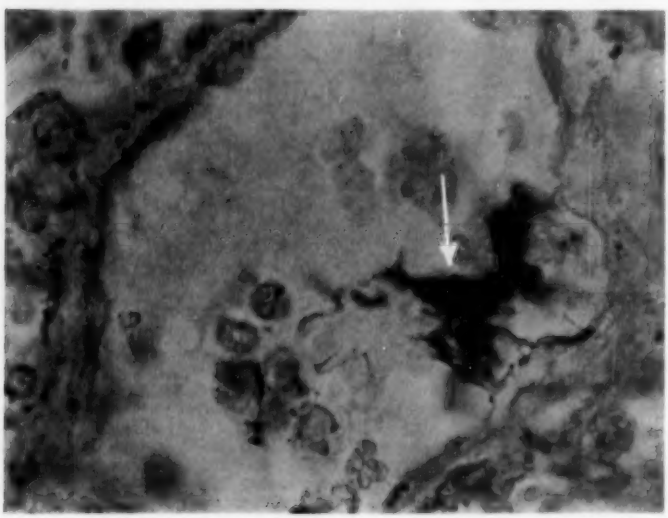

Fig. 2—Hamster lung from twentieth passage, died three days following infection. The cross section of a vein contains a leptospiral embolus (arrow) and also a few individual Leptospira. Warthin's silver stain; x 1,725.

After several hamster passages, the virulence of the organisms tended to decline for cattle. This may be comparable to the attenuation of hog cholera virus by serial passage in rabbits as reported by Baker.[1] He found that after continued serial passage the virulence of the virus for swine was reduced while the antigenic properties were retained. The Leptospira tended to increase in virulence for hamsters as the passage progressed but appeared to lose its virulence for cattle. It would appear that this strain became adapted to hamsters and, at the same time, caused only a transient febrile response in cattle. This was particularly evident in bull 700 when infective tissues of the twenty-third hamster passage were used for exposure. The strain probably became incapable of producing any clinical evidence of the disease but retained its antigenicity as proved by serological tests. Brown *et al.*[2] reported similar observations in which the virulence of a certain strain of *L. pomona* had been altered for cattle after continued passage through guinea pigs.

Bull 28 exhibited an unusual resistance to leptospiral infection as evidenced by the failure to isolate the organisms from his blood; also, the first appearance of antibodies did not occur until 27 days following exposure. This animal was calved by a cow which had previously been experimentally infected with *L. pomona*. The bull obtained natural passive immunity from his dam's colostrum and milk during the first week of his life. However, he was negative for the agglutination-lysis test when exposed at 5 months of age. This unexpected resistance might be due to either individual natural resistance or to an undetected active immunity induced by contact with leptospiras *in utero* or from his dam's urine during his early life.

Bull 1161, which was infected with hamster tissue of the thirty-ninth passage of the second series, showed a febrile response of four days' duration. He was 2 months old when exposed with the same amount of infective material as that given to adult animals. This febrile response was the only clinical manifestation of the disease and he recovered promptly. Webster and Reynolds [14] reported that the acute clinical syndrome of leptospirosis is much more pronounced in young animals. This might explain the febrile reaction of the calf, even though he was exposed to a well-adapted hamster strain of *L. pomona*.

Summary and Conclusions

Leptospira pomona (Hardacre strain) was maintained in hamsters by direct animal passage using infected blood. In the early passages, the hamsters were readily infected; mortality was low but, when the hamsters were killed up to 84 days later, the kidneys were grossly involved and leptospiras were demonstrated by dark field microscopy.

The strain increased in virulence as the passage progressed: at the sixteenth passage, the hamsters died within four days; by the twentieth passage, all died on the third day. However, at the twenty-sixth passage, the virulence appeared to decline; the hamsters died after six days.

In a second serial passage, hamsters started dying by the fifth day after exposure at the twenty-ninth passage with mortality approximating 100 per cent. At the fifty-first passage, 0.2 ml. of a 10^{-9} dilution produced death within 11 days while a 10^{-10} dilution was not infective. In both trials, the number of leptospiras in the inoculum and probably the ages of hamsters appeared to be factors in the decline of virulence.

Tissues from the seventh hamster passage caused severe leptospirosis in 2 young bulls and a pregnant heifer. The strain appeared to lose its virulence for cattle after 23 or more serial passages through hamsters; 3 young bulls developed only mild clinical signs.

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Experimental Bovine Leptospirosis

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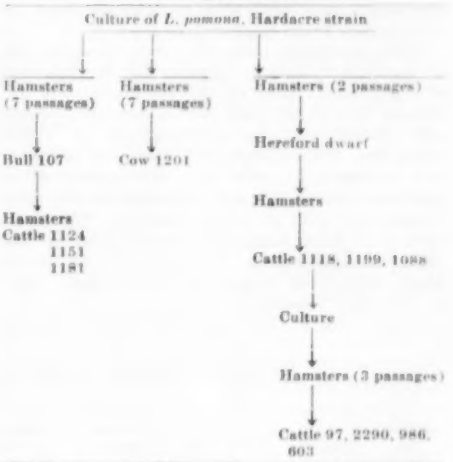
THE FIRST detailed report of bovine leptospirosis in the United States was that of Jungherr [1] in 1944. Baker and Little [2] reported the first isolation of the causative agent of leptospirosis of cattle in this country and subsequently the organism was identified as *Leptospira pomona*. The importance of leptospirosis in cattle has been confirmed by reports from various parts of the country. Bohl and Ferguson [3] reported that 3.5 per cent of 352 cattle picked at random in Ohio were positive serologically for *L. pomona*. In Illinois, 11.2 per cent of 15,018 cattle in 1,528 herds were found to be positive serologically.[4] Stoenner [5] reported that, of 7,066 cattle in 298 herds located in Montana, Idaho, Washington, and Oregon, about 11.0 per cent were positive in tests with *L. pomona*. In Ohio, and probably in other states as well, practicing veterinarians are reporting increasing numbers of abortions in cattle occurring in serologically positive herds. A report of the U. S. Department of Agriculture in 1954 [6] estimated the losses from leptospirosis in cattle in excess of 112 million dollars annually.

The increasing losses from leptospirosis led to the experiments reported in this paper. The objectives were to determine the factors involved in reproductive failure and pathological changes which occur in infected cattle and aborted fetuses.

MATERIALS AND METHODS

The strain of *L. pomona* used in this experiment was isolated * approximately six months prior to the beginning of this experiment from the urine of a cow exposed during a naturally occurring outbreak of leptospirosis. In this herd of 47 cows, there were 16 abortions during a period of three months. The organism, isolated from hamsters which had been injected with urine from the infected cow, was maintained in Schüffner's medium. Just prior to its use in this experiment, the strain was passed through 2 hamsters in series and blood cultures from the second hamster were used as the source of *L. pomona*. The history of the inoculum for each group of animals challenged with *L. pomona* is given (table 1). The serial passages in hamsters were carried by collecting 0.1 to 0.5 cc. of heart blood from the infected hamster on the fourth day and injecting it intraperitoneally into a susceptible hamster. Infected hamsters were killed, usually on the fourth day, and blood, liver, spleen, and kidneys from each hamster were ground in a TenBroeck tissue grinder with 10.0 cc. Schüffner's medium. Five cubic centimeters of the homogenized tissue was injected intramuscularly into each cow.

TABLE 1—History of Leptospira Pomona Used to Expose Cattle



Leptospira pomona, strain NIH, was maintained by cultivation in Schüffner's medium. This strain was used as the antigen in the agglutination-lysis test after growing three to seven days at 30 C. In the latter part of these experiments, cultures which had grown more than three days were diluted with Schüffner's medium to approximate the concentration of leptospiras in a three-day

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* This strain was obtained from Drs. E. H. Bohl and T. E. Powers, Department of Bacteriology, Ohio State University, Columbus.

culture.[7] The serum to be tested was diluted in a saline solution and equal volumes (0.1 ml.) of the diluted serum and culture were incubated at room temperature for one to three hours. A loopful of the mixture was examined on a slide with a dark field microscope at a magnification of x 150. The titer of a particular serum sample was designated as the highest dilution of serum in which 50 per cent or more of the leptospiras were agglutinated or lysed.

The cattle used in this experiment were chiefly Holstein-Friesian or Jersey breeds; 1 was a crossbred Hereford. None were lactating. The breed, age, and sex of each animal are given in table 2.

Blood was collected daily from the cattle during the acute phase of leptospirosis and subsequently at weekly or monthly intervals. Samples for blood cultures were collected in a sterile syringe from the jugular vein and 0.1 to 0.5 ml. was introduced into each of two to four tubes of Schüffner's medium. These cultures were usually examined at weekly intervals by dark field microscopy and were held at least 30 days at 30 C. before being discarded as negative.

Urine specimens from the cattle were checked at intervals following exposure for the presence of leptospiras. Some of the specimens were examined directly by dark field microscopy; others were concentrated by centrifugation at 10,000 r.p.m. for five minutes and then the sediment was examined by dark field microscopy; other specimens were injected intraperitoneally into hamsters in 1.0- or 2.0-ml. amounts. The hamsters were bled aseptically from the heart four days following injection and 0.1 to 0.5 ml. of the blood was placed in each of two or more tubes of Schüffner's medium.

Kidney tissue from cows which were slaughtered following infection and various tissues from aborted fetuses were examined for the presence of leptospiras by hamster inoculation. Blood was collected from the hamsters on the fourth day and cultured in Schüffner's medium.

Tissues collected from aborted fetuses and kidney tissue from the cows at slaughter were fixed in neutral buffered formalin, sectioned at 5 μ and stained with hematoxylin and eosin, Warthin's silver-impregnation method, or other special stains where needed.

RESULTS

The observations made on 12 cattle exposed to *L. pomona* are summarized (table 2).

A culture of *L. pomona* was injected into hamsters and it was maintained by direct serial passage.[8] Liver and blood from the seventh hamster passage were ground in Schüffner's medium and 5.0 cc. was injected intramuscularly into a yearling bull, 107 (table 1). The bull was febrile on the third day and the high temperature persisted to the sixth day but the maximum was 105.4 F. Anorexia was evident on the fifth and sixth days. On the morning of the seventh day, the bull was unable to rise without help and walked with a staggering gait. The temperature was subnormal, the pulse was weak but rapid, and the respirations were rapid and labored. Incontinence and a reddish color of the urine were noticed. Death occurred on the seventh day.

TABLE 2—Response of Cattle to Infection with Leptospira Pomona

Animal No.	Breed	Sex	Age (yr.)	Day[1] of febrile response	Day of leptospiremia	Maximum titer[2] Day	Maximum titer[2] Level	Day of leptospiruria	Results of pregnancy at postinfection days
107	Jersey	M	1	3, 4, 5	3, 4	7	160[3]	....[4]	.....
1124	Jersey	F	3	3, 4	2, 3	13	25,600	—	Aborted 7-month fetus at day 19.
1151	Holstein-Friesian	F	3	1	2	15	25,600	18	Normal pregnancy, calved day 63.
1181	Jersey	F	2	3, 4, 5	1, 2, 3, 4, 5	13	6,400	18	6-month pregnancy, cesarean section.
1201	Jersey	F	2	4, 5, 6	1, 2, 3	9	2,560	19, 21, 23, 25	5½-month pregnancy, aborted at day 20.
1118	Holstein-Friesian	F	4	1	—[5]	25	102,400	—	Normal calf on day 22.
1088	Holstein-Friesian	F	5	—	—	25	102,400	53, 102	Calved normally on day 49.
1199	Jersey	F	2	5	4, 5	25	102,400	—	Aborted a 6-month fetus at day 47.
97	Jersey	M	1	3, 4, 6	2, 3, 4	20	102,400	13, 24	.....
2290	Holstein-Friesian	F	4	4, 5	2, 3, 4, 5	20	102,400	24, 34	Fetus normal at day 98, laparotomy performed.
603	Jersey	F	4	3, 4	2, 3	20	102,400	—	Calved normally day 179.
986	Holstein-Friesian	F	7	5	2, 3, 5, 6	20	102,400	24	Not pregnant.

[1] "Day" refers to the number of days following the artificial exposure to *L. pomona*. [2] The titers are expressed as the reciprocal of the highest dilution of serum in which one half or more of the leptospiras were agglutinated or lysed. [3] Titer at the time of death on seventh day. [4] *Leptospira pomona* was isolated from the kidney after death. [5] — indicates that the observations were negative or were not made.

Necropsy revealed no outstanding gross lesions other than slight icterus in the brain and liver with petechiae in the subcutaneous tissues and kidneys. The bladder contained about 1 liter of reddish urine. The blood appeared watery. *Leptospira pomona* was recovered from the kidneys but not from the liver by hamster inoculation.

Histological sections were made of many tissues but only the kidney, liver, spleen, and adrenal showed any significant changes. In the kidneys, nearly every glomerular space contained some granular, eosinophilic material and, in addition, fibrin and erythrocytes were present in some. The capillary tufts were in various stages of degeneration with some appearing as broken fragments and others as shrunken dense clumps. The epithelium of the proximal convoluted tubules was absent in some places and undergoing necrobiosis in others. Many tubules contained eosinophilic granular material, fibrin, and erythrocytes. Numerous tubules were filled with heme casts. Tubules were completely destroyed in some places but occasionally remnants of epithelial cells remained. Some collecting tubules contained erythrocytes or hemoglobin. Many cells in the collecting tubules showed hydropic degeneration. Lymphocytes and monocytes had invaded some areas.

Centrolobular necrosis of the liver was widespread. Lobule outlines were indistinct, sinusoids were partly obliterated, cytoplasm of cells was confluent, and nuclei were pyknotic or undergoing karyorrhexis. Many hepatic cells contained large clear vacuoles. Lymphocytes and plasma cells were present in the interlobular spaces and around bile capillaries.

Massive hemorrhage had occurred in the spleen. Only occasional germinal centers could be seen and scattered reticuloendothelial cells were present. The adrenal glands contained petechiae in the medulla. Silver stains failed to reveal leptospiras.

Blood from bull 107, collected four days after infection, was injected into hamsters. Four days later, livers and blood from 2 hamsters were ground in Schüffner's medium and 5.0 cc. of the suspension was given intramuscularly to each of 3 cows, 1124, 1151, and 1181. All the cows developed a fever and leptospiras were isolated from each on one or more days during the first week (table 2). Leptospiras were demonstrated in the urine of 2 of the cows by hamster inoculation.

Cow 1124, carrying a 7-month fetus when infected, showed some indication of impending abortion on day 18. The udder had developed in size and there seemed to be some relaxation of the pelvic region. By rectal palpation, the uterine artery felt normal for this stage of pregnancy but whether the fetus was living could not be determined. The following morning the well-developed dead calf was found in the pen. Its thymus was enlarged to the extent that the cervical region appeared distended. The pleural cavity contained about a pint of fluid which was deeply stained with hemoglobin but no erythrocytes were present. The heart and lungs appeared normal. The spleen was greatly enlarged and congested; in fact, this was the only part of the body where erythrocytes were evident. The liver possessed a yellowish color on cut section. The kidneys were soft and "pulpy." Spleen, pleural fluid, blood, and aqueous humor were cultured in Schüffner's medium and injected into hamsters. All were negative for *L. pomona*.

Cow 1151 was just over seven months in gestation when exposed to *L. pomona*. Her pregnancy progressed normally and a healthy heifer calf was delivered at term.

Cow 1181 was carrying a 6-month fetus when exposed to *L. pomona*. On postinfection day 13, a cesarean section was performed using local anesthesia with the cow standing. A living, well-developed, female fetus was removed. Upon necropsy, its stomach was distended with about 1 liter of yellowish, thin fluid. Fetal fluids, stomach contents, blood, and liver tissue were cultured in Schüffner's medium and also injected into hamsters. No leptospiras were demonstrated from any of the tissues.

Cows 1181 and 1124 were slaughtered four months following infection. Grossly, the kidneys from cow 1181 were negative for lesions but a healed infarct was present in one kidney from cow 1124.

Cow 1201, about 5½ months pregnant when exposed to *L. pomona* (table 2), aborted a well-developed dead fetus 20 days later. The peritoneal and pleural cavities contained large amounts of fluid which was dark red but contained no erythrocytes. Hamsters and Schüffner's medium were inoculated with pleural fluid, peritoneal fluid, stomach contents, and ground

liver and kidney tissue, but no leptospiras were demonstrated. Tissues were not available for study from cow 1201.

Cows 1118, 1088, and 1199 were exposed to *L. pomona* as shown in table 1. Cows 1118 and 1088, which were nearing the end of pregnancy when exposed, were never clinically ill and no leptospiras were demonstrated in the blood, but both were infected as indicated by the appearance of antibodies for *L. pomona* and by the presence of leptospiras in the urine of 1088 on postexposure days 53 and 102.

Cow 1199, which developed a more classic picture of leptospirosis, had a definite febrile response on day 5, a leptospiremia on days 4 and 5, showed antibodies on day 7, and reached a maximum titer of 1:102,400 on day 25. Pregnancy in this cow had progressed to about five months at the time of infection, and abortion occurred 47 days later. Degenerating fetal membrane was seen protruding from the vulva and, by manipulation, the unbroken membrane containing the dehydrated fetus was removed even though the cervix was only partially dilated. The fetus measured 8½ inches from crown to rump. It appeared to have been dead for two weeks or more. No blood or other body fluids were present. Portions of fetal liver, stomach, heart, and lungs were ground in Schüffner's medium and injected into 4 hamsters. No leptospiras were demonstrated in the fetus either directly or in the inoculated hamsters.

These 3 cows were slaughtered four months following infection and the kidneys were obtained for study. There were no visible gross lesions in the kidneys of cows 1118 and 1199, but one kidney from cow 1088 was greatly enlarged and had numerous small white foci scattered over the surface.

Four cattle, bull 97 and cows 2290, 603, and 986, were exposed with a strain of *L. pomona* derived from a blood culture of 1199 (table 1). All four of the animals (table 2) developed a fever at three to six days following infection which lasted for one or more days. *Leptospira pomona* was isolated from the blood of all 4 animals on two or more days during the first week of the disease. Antibody production was first detected in daily serological tests on postinfection days 5 or 7 and a peak titer was demonstrated on day 20 at a serum dilution of 1:102,400. Leptospiras were demonstrated in the urine of 3 of the 4 animals between days 13 and 34 by hamster inoculation.

Cow 2290 was at the twenty-ninth day of gestation when infected and the pregnancy appeared to progress normally. When gestation had progressed to 127 days, laparotomy was performed and 5.0 cc. of ground liver tissue from an infected hamster, twenty-first passage, was injected with a 20-gauge needle through the uterine wall into the cervical region of the fetus. Prior to this treatment, cow 2290 had an antibody titer of 1:3,200 and during the week following the treatment, no anamnestic response was observed. On postoperative day 7, a purulent exudate was observed and examination revealed that a decomposing fetus was being aborted. Dark field examination of the exudate revealed dead leptospiras plus motile rods and cocci. *Escherichia coli* and streptococci were cultured from the exudate. Hamsters given some of the exudate intraperitoneally died within two days from a purulent infection and no leptospiras were demonstrated.

Cow 603 was exposed to *L. pomona* at 101 days of pregnancy. No clinical abnormalities were observed and a normal calf was born at term. Tissues from this cow were not available for study.

Cow 986 was inseminated 15 days prior to infection with *L. pomona* and, although no signs of estrus were noticed, subsequent rectal examination revealed no pregnancy. The level of antibodies had declined to 1:800 at three months following infection and she was sent to slaughter.

The kidneys from bull 97 were negative for gross lesions. The kidneys from cow 2290 contained small subcapsular cysts and numerous grayish foci. One kidney from cow 986 contained a large encapsulated abscess and several small abscesses. Adhesions extended from this kidney to the liver, omentum, and diaphragm. An organism resembling *Corynebacterium pyogenes* was isolated from the abscess and kidney tissue. The possible presence of leptospiras was obscured by the pyogenic infection which caused early death of the hamsters which were inoculated with kidney tissue.

Histopathology.—Histopathological findings were similar in all the kidneys that were studied. Variations were mainly in degree and the observations on the chronic cases will be described together.

Both in the cortex and medulla, much of the tubular epithelium was undergoing degeneration. Many cells were absent. Others had broken cell membranes which allowed cytoplasm and nuclei to escape into the lumen, while in some tubules the epithelium was shrunken and pulled away from the basement membrane.

The abscessed kidney of cow 986 was not studied but the kidney which contained no abscesses had lesions similar to those described for the other animals. In addition, some tubules were lined with double rows of epithelium suggesting hyperplasia. In other kidneys, some tubules contained large, deeply basophilic cells attached to the basement membrane, indicating regeneration.

Many cortical and medullary tubules in all kidneys contained granular, eosinophilic debris or hyaline casts. Occasionally, broken tubules were seen. In most of the kidneys, there were dense infiltrations of lymphocytes primarily in the cortex. Within these lymphocytic foci, remnants of tubules and a few intact tubules remained although most of the parenchyma had been destroyed. In cow 1088, the dense lymphocytic infiltration ended abruptly at the corticomedullary junction. Numerous tubules were filled with free blood or contained heme casts. Glomeruli were in progressive stages of degeneration. Some capillary tufts were congested; others were atrophic, dense, and deeply basophilic; in some, only fragments of the capillary loops remained; while in some glomeruli, the entire tuft had been destroyed. Bowman's spaces contained erythrocytes, debris, and fragments of epithelial cells. The parietal layer of some of the Bowman's capsules was thickened and dark-pigmented material was present in some spaces as well as in some tubules. Iron stain identified this as hemoglobin.

In cow 2290, there were numerous subcapsular cysts formed from widely dilated, thin-walled tubules. Adjacent to these areas the tubules and glomeruli were atrophic, lymphocytes had infiltrated, and fibrous tissue was noticeably increased.

Silver stains failed to reveal leptospiras in these kidneys.

Discussion

All 12 cattle exposed in this experiment became infected with *L. pomona*. Of the 12 animals, 11 developed a leptospiremia during one or more days of the first week. The 1 cow from which negative blood cultures were obtained did shed leptospiras in the urine at a later time. In 8 of the 12 cattle, the leptospiras localized in the kidneys as evidenced by isolation of *L. pomona* from the urine following inoculation of hamsters.

The above facts, plus the death of 1 (bull 107) with typical acute, fatal leptospirosis, confirm the virulence of the strain of *L. pomona* used in this experiment. Further consideration of the factors involved in this virulence of *L. pomona* are presented in another paper.[8]

Of the 9 pregnant cows studied, 3 aborted fetuses between postexposure days 19 and 47. This proportion of abortions is similar to that which has been observed in cattle exposed during naturally occurring leptospirosis. All 3 of the cows which aborted were of the Jersey breed. This might suggest a greater susceptibility of this breed than of Holstein-Friesian cattle; however, the numbers are too small to justify a definite conclusion.

The basic cause of abortion in cattle infected with *L. pomona* remains obscure. In swine leptospirosis, the organism can be demonstrated rather readily in living, weak pigs aborted or farrowed by an infected sow. However, the Podgwaite *et al.* report [10] is the only one seen by the authors on the isolation of *L. pomona* from an aborted bovine fetus. The methods of isolation or the nature of the disease present in that case might have been different from those of other workers. In most, if not all, of the other reported cases of abortion in cattle caused by *L. pomona*,[11,12] the abortion occurred two to six weeks after the acute phase of leptospirosis in the dam when the antibody titer for *L. pomona* was at or near its peak. Since Podgwaite *et al.*[10] reported that 2 of the 3 cows were negative, serologically, for *L. pomona* at the time of abortion but positive (1:100) one and four months later, one may conclude that they were not dealing with typical leptospirosis.

A second irregularity was the isolation of *L. pomona* from the liver of their third cow one month following abortion. This cow, according to the report,[10] presented a typical course of leptospiral infection since she possessed a titer for *L. pomona* of 1:

8,000 at the time of abortion but it had fallen to 1:1,000 at the time of slaughter one month later. The demonstration of *L. pomona* in tissues other than the kidneys of animals in the chronic phase of leptospirosis deviates from all other published reports which we have seen.

Even though there is adequate evidence that *L. pomona* causes abortion in cattle, the exact mechanism remains obscure. Cesarean section was performed on cow 1181 in an attempt to shed some light on this mechanism; however, the results were essentially negative. This cow developed a leptospiremia on the first day after exposure and it persisted through the fifth day. Antibodies for *L. pomona* were present on the seventh day at a level of 1:800. One possible explanation of the failure to demonstrate leptospiras in the aborted fetus is that the organisms might be present in the fetus and cause its death but, prior to abortion, the leptospiras die in the unfavorable environment in the dead tissues. It was assumed in the case of cow 1181 that if the leptospiras had actually invaded the fetus the living organisms should still be present on the thirteenth day. Our failure to find leptospiras either by dark field microscopy, by direct culture, or by hamster inoculation leaves little question that the organisms were not present in this fetus. It is possible, of course, that this cow would not have aborted had she been permitted to continue the pregnancy. Certainly, this approach to the problem needs repetition, but this single observation offers some evidence that *L. pomona* does not regularly invade the fetus in the pregnant cow.

Another approach to the question of the effect of *L. pomona* on the fetus was that involving cow 2290. She had passed through a rather typical form of leptospirosis with a febrile response, leptospiremia, and leptospiruria. However, at 98 days following exposure, the titer had receded to 1:3,200 and the fetus was living. Virulent *L. pomona* in hamster tissue suspension was injected directly into the fetus through the uterine wall. Unfortunately, the tissue suspension was contaminated with other bacteria and abortion followed, together with nearly complete decomposition. This type of experiment should be repeated, for by this method the effect of *L. pomona* on the fetus could be determined directly irrespective of the immune status of the dam.

The apparent lack of erythrocytes in the heart, blood vessels, and other tissues of the aborted fetuses seen in this experiment, as well as those observed in naturally occurring leptospirosis, may be of significance. Unpublished observations in both infected cattle and swine indicate that the erythrocyte count remains at a normal level until about the time antibodies for *L. pomona* appear in the circulating blood. Following this, there is frequently a decrease in the count followed by icterus in the severe cases. This destruction of erythrocytes may result from the presence of a toxic substance released from the organism at the time when large numbers of the leptospiras are destroyed by the developing antibodies.

This explanation may be applied to the observations made on the aborted fetus. Thus, it is proposed that even though the leptospiras may not pass the placental barrier, the soluble toxic product from the dead leptospiras does pass into the fetus. The erythrocytes of the fetus, it is proposed, are destroyed in such quantity that anoxia causes death which is followed by abortion.

Histopathological lesions in the experimental disease closely resembled those described by Jungherr,[1] Cordy and Jasper,[13] and Matthews[14] in naturally occurring cases. The principal lesions included hemorrhage, centrolobular hepatic necrosis and necrobiosis, atrophy, fibrosis, and lymphocytic infiltration in the kidneys.

SUMMARY AND CONCLUSIONS

All of 12 cattle exposed to *Leptospira pomona* by intramuscular inoculation developed leptospirosis. One of these, a yearling bull, became seriously ill and died on the seventh day. The lesions in this animal were typical of those commonly seen in leptospirosis.

Of the 9 pregnant cows, 3 aborted fetuses on days 19, 20, and 47 following exposure to *L. pomona*.

Histopathological lesions in the experimental disease closely resembled those described in the naturally occurring disease in cattle.

The presence of a toxic substance, released from the leptospiras when large numbers are destroyed by antibodies, is

proposed. This substance may cause the destruction of erythrocytes in the cow and, further, it presumably passes the placental barrier destroying some or all of the red blood cells of the fetus. This latter effect is proposed as the explanation for abortion of the bovine fetus in which no leptospiras are demonstrated.

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The Presence of *Leptospira Sejroe* Agglutinins in Cattle Serum

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SEROLOGICAL EVIDENCE of the existence of *Leptospira sejroe* in cattle has been reported from several states.[1-3] This strain has never been isolated from naturally occurring cases of leptospirosis in the United States but was originally reported from Denmark by Borg-Peterson as occurring in both mice and man.[4] In our own laboratory, using the microscopic agglutination-lysis test with living leptospiral antigens,[5] serums from many herds of cattle have been found to have high titers against the test serotype *L. sejroe* (Mallersdorf strain *). In most herds, agglutinins have been detected for this leptospiral serotype alone and clinical histories in the cattle herds where the serum originated have included such symptoms as abortions and sterility. In other herds where *Leptospira pomona* had been isolated or incriminated serologically (by serial serum titer increases), concurrent infection with *L. sejroe* apparently occurred as evidenced by the presence of serum agglutinins.

Wolf [6] has shown that *L. sejroe* is serologically unrelated to *L. pomona*. Therefore, it could be assumed that separate antigenic entities are responsible for *L. pomona* and *L. sejroe* serum agglutinins in cattle.

However, three postulates presented as possible explanations for the existence of *L. sejroe* agglutinins in certain bovine serums were: (1) that such serum reactions were false responses due to such diseases as vibriosis and brucellosis which could cause clinical symptoms similar to those found in bovine leptospirosis; (2) that leptospiras of another serotype, enzootic in wildlife,[7] such as *Leptospira ballum*, could upon infecting or entering the bovine animal cause agglutinins against *L. sejroe*—Broom [8] suggested the possibility of strain mutation and this was considered a possibility in the light of the existence of *L. ballum* in wildlife infesting grazing areas where cattle were serologically reactive against *L. sejroe* antigens; and (3) that *L. sejroe* alone was responsible for the existence of agglutinins against this serotype.

This work was preliminary to proving or disproving postulates 1, 2, and 3.

TABLE 1—Serum Agglutination-Lysis Titers of Calf 7 Inoculated with Leptospira Ballum

Day	Antigens		
	L. ballum	*L. pomona*	*L. sejroe*
D	0	0	0
D + 5	1:1,600	0	0
D + 10	1:1,600	0	0
D + 19	1:6,400	0	0
D + 21	1:6,400	0	0
D + 25	1:25,600	0	0
D + 27	1:25,600	0	0

EXPERIMENTAL PROCEDURE

Serums were obtained from 25 cattle positive to the test for brucellosis and were tested against 16 leptospiral serotype antigens: *L. andaman*, *L. semerang*, *L. ERR*, *L. bataviae* SVT, *L. pomona* S91, *L.djasiman*, *L. sentot*, *L. autumnalis akiyamia*, *L. ballum*, *L. canicola*, *L. icterohaemorrhagiae* W, *L. pyrogenes* S, *L. alexi* Z, *L. grippotyphosa* MV, *L. sejroe* M, and *L. hebdomadis*.†

Serums were obtained from another 20 cattle which had been either experimentally or naturally infected with pathogenic *Vibrio fetus*. These were also tested against the above 16 leptospiral serotype antigens. The microscopic agglutination-lysis test, using living antigens, was employed for the serological test.

Leptospira ballum was grown in Stuart's medium for five days and 20 ml. of this material containing large numbers of living leptospiras was inoculated intramuscularly into an experimental dairy calf, 6 weeks old. The calf's temperature was taken twice daily throughout the

† Strain cultures originally obtained from Veterinary Division, Walter Reed Army Institute of Medical Research, Washington, D.C.

TABLE 2—Serum Agglutination-Lysis Titers of Calf 29 Inoculated with Leptospira Sejroe

Day	Antigens		
	L. ballum	*L. pomona*	*L. sejroe*
D	0	0	0
D + 5	0	0	1:100
D + 10	0	0	1:400
D + 19	0	0	1:1,600
D + 25	0	0	1:6,400
D + 30	0	0	1:6,400

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* Obtained from Veterinary Division, Walter Reed Army Institute of Medical Research, Washington, D.C.

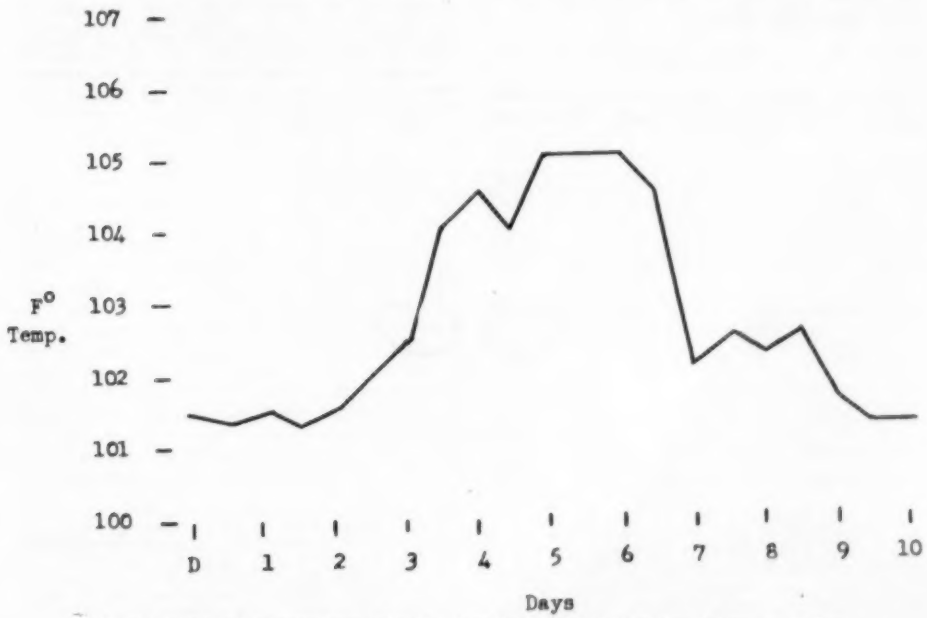
Fig. 1—Kidney of calf 7 inoculated with Leptospira ballum 27 days previously. Note the numerous white foci.

study (27 days), after which the animal was slaughtered for necropsy. A similar procedure was used with *L. sejroe* (Mallersdorf) in another experimental calf.

RESULTS

All serums giving positive reactions against *Brucella abortus* or *V. fetus* antigens were negative against the 16 leptospiral antigens. It was fortunate that serum obtained and so tested was not from cattle concurrently infected with brucellosis, vibriosis, and leptospirosis. Although many of the serums had high agglutinin titers (1:200 or greater) against Brucella tube antigens none cross reacted with any of the leptospiral agents.

The calf experimentally inoculated with *L. ballum* exhibited a clinical reaction of fever and anorexia (graph 1). Serological response to the inoculation indicated the presence of agglutinins solely against the serotype *L. ballum* (table 1). Upon necropsy (27th day), kidney lesions typical of leptospirosis were found (fig. 1). The calf inoculated with *L. sejroe* exhibited no observable clinical signs of leptospirosis but, serologically, it was possible to



Graph 1—Rectal temperatures of calf 7 inoculated with Leptospira ballum on D-day.

demonstrate a serum-agglutinin titer rise against the homologous leptospiral serotype (table 2). Necropsy findings on this animal were insignificant.

DISCUSSION

It is indicated that *L. sejroe*, although never bacteriologically proved to exist in this country, is apparently necessary for the presence of agglutinins against this serotype. This work has been in the nature of a pilot study but helps support certain other research conducted on a broader scale in which bovine agglutination-lysis titers against *L. sejroe* have been confusing.[9]

Leptospira sejroe has never been isolated from naturally occurring cases of leptospirosis, perhaps due to a lack in techniques which are successful for isolation of other leptospiral serotypes. Laboratory animals, such as hamsters and guinea pigs, ordinarily used for isolation purposes are not often suitable for use with this serotype. *Leptospira sejroe* has not in most cases, been recovered or blind-passaged in such animals after experimental inoculation with cultures of the organism.[9] Other techniques may have to be applied before this serotype will be isolated from naturally occurring cases.

SUMMARY

Bovine serums containing agglutinins against *Brucella abortus* and *Vibrio fetus* were tested against 16 leptospiral antigens (living cultures) in the microscopic agglutination-lysis test. These serums did not give positive reactions against any of the leptospiral antigens.

Leptospira ballum and *Leptospira sejroe*, when inoculated separately into 2 calves, each produced agglutinins against the homologous strain antigen but not against the other antigen.

It is suggested that the presence of *L. sejroe* agglutinins in bovine serums is due to exposure to *L. sejroe* and that eventual bacteriological proof will substantiate such serological evidence of the existence of this leptospiral serotype in the United States.

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Histopathological Alterations of the Bovine Uterus
I. Studies with *Vibrio Fetus*

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THE ROLE of *Vibrio fetus* in bovine reproductive failures has not been established accurately. Similarly, the changes within the infected uterus have not been fully elucidated and the significance of the serum-agglutination reaction for vibrionic infection is not entirely clear. In an attempt to gain more knowledge on these and other questions, the disease in experimentally infected heifers was studied.

REVIEW OF LITERATURE

McFadyean and Stockman,[1] of England, apparently first recognized *V. fetus* infection as an epizootic of abortions in ewes. Others[2-6] have reported ovine *V. fetus* infection. According to these reports, over 50 per cent of ewes aborted in some flocks.

Smith[9,10] described 14 cases of vibrionic abortion in cows and pointed out that only Vibrio organisms could be demonstrated. He examined 109 aborted bovine fetuses and found Vibrio in 26 or 23.8 per cent. Smith[12] and Smith *et al.*[13] described their studies on the etiological role of *V. fetus* and on acquired immunity in vibrionic abortion. Smith[14] was able to infect guinea pigs and cattle with *V. fetus* by artificial exposure. Lerche[15] examined 1,565 aborted bovine fetuses and found 22 or 1.41 per cent were due to vibriosis. Others[16-23] have reported *V. fetus* infection in cattle. Stegenga[24] investigated the relationship of *V. fetus* to "enzootic sterility" and concluded that vibriosis is the cause of enzootic sterility.

Blakemore and Gledhill[25] used one strain and Levi[26] used five strains of *V. fetus* of ovine origin for the serum-agglutination test for vibrionic abortion of sheep. Both authors concluded that the agglutination test had little diagnostic value. Smith *et al.*[13] obtained variable results with the serum-agglutination test for *V. fetus*. Plastridge[27] considered a serum-agglutination titer of 1:200 or higher as a positive indication of vibriosis in cattle, 1:100 as suspicious, and 1:50 as insignificant. Brocklehurst[28] considered agglutination in a 1:320 dilution or higher of the serum as positive for vibriosis. Plastridge and Williams[29] concluded that an agglutination at serum dilution of 1:400 or higher should be considered positive for prior vibriosis in cattle. Moore[30] believed that vibriosis in cattle was prevalent in the United States because a large number of cattle reacted when tested by the agglutination test. Roberts *et al.*[31] questioned the reliability of the agglutination test for vibriosis. According to Brus,[32] the serum-agglutination test for *V. fetus* is unreliable. He suggested the use of either cervical or uterine secretions in a tube-agglutination test. Sato *et al.*[33] studied the agglutinins and chemical composition of the colostrum of a cow and found agglutinins for *Escherichia coli* and *V. fetus* and, to a lesser degree, for *Salmonella paratyphi* B, *Brucella melitensis*, and *Brucella abortus*.

Gardner and Venkatraman[34] defined the cholera group of vibrios as consisting of vibrios with similar biochemical characters and a common heat labile antigenic component.

McCullough *et al.*[35] demonstrated the antigenic relationship of Brucella and *Vibrio cholerae* by reciprocal agglutinin absorption tests with antiserums prepared against *V. cholerae* antigens.

MATERIAL AND METHODS

The vibrios were grown as follows. Stock cultures in semisolid Albimi Brucella broth were examined for motility and purity. Of each culture, 0.5 ml. was inoculated into 10.0 ml. of sterile Brucella broth (Albimi) reinforced with 0.1 per cent agar (semisolid), the pH of which had been adjusted to 6.8. The inoculated medium was incubated under 10.0 per cent CO_2 at 37° C. for 48 hours. Then 1.0 ml. of each broth culture was seeded upon bottle slopes of Brucella agar (Albimi) which was reinforced by the addition of 1.0 per cent agar, so that the final agar concentration was 2.5 per cent and adjusted to a pH of

TABLE 1—Vibrio Fetus Strains* Used in Experimental Studies

Strain designation	Source of strain
543	Bureau of Animal Industry; ovine origin.
M_3	Isolated from stomach contents and liver of aborted bovine fetus in Wisconsin, April, 1950.
P_1	Culture Longo (from W. N. Plastridge, 2/24/49).
R_2	Bureau of Animal Industry; bovine origin.
M_2	Isolated from stomach contents of aborted bovine fetus in Illinois, July, 1949.
D_{77}	Bureau of Animal Industry; bovine origin.

* The cultures of vibrios used in this study were obtained from Mr. Gordon Roberstad, Department of Veterinary Science, University of Wisconsin.

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TABLE 2—Tube-Agglutination Test Titers with Vibrio Fetus Antigens of Serums from Heifers Prior to Experimental Inoculations

Heifer No.	Antigens					
	543	R_5	M_5	M_2	P_1	D_{71}
697	1:80					
51712	1:160					1:20
684	1:80					
53	1:80					
1	1:80		1:40			
691	1:80					
31312	1:160					
13Z	1:40				1:20	
694	1:320				1:80	
31313	1:80					1:40
989	1:160	1:20				1:80
47824	1:160					
89030	1:160					
31315	1:80					
2	1:160				1:80	
89002	1:80					
34471	1:80	1:10				

6.8. The inoculated slope cultures were incubated at 37 C. under 10.0 per cent CO_2 for 72 hours.

The slopes for the first series of inoculations contained 200.0 ml. of agar in Roux bottles. Since this size bottle was cumbersome and frequently resulted in contamination, smaller bottles containing 50.0 ml. of agar were used for subsequent experiments.

The fastidious growth requirements and great motility make it extremely difficult, if not impossible, to use plate counts or hemocytometer counts to estimate the number of cells in actively growing *V. fetus* cultures. In addition, growth of the Vibrio cultures varied considerably, making standardization of inoculums difficult.

The growth upon the inoculated slopes was washed off with 10.0 ml. of sterile distilled water adjusted to a pH of 6.8. The cell suspensions were poured into sterile inulin tubes and motility and apparent purity checked by examination of hanging drop preparations. A final mixed culture of 10.0 ml., approximating the turbidity of the tube 7, McFarland nephelometer, was prepared and used for the inoculations.

The cattle used in this study comprised a self-contained group which was negative to the agglutination test for brucellosis. All were virgin heifers, mainly of the Holstein-Friesian breed, in excellent physical condition, and maintained under excellent sanitary conditions and management.

The serums of all animals were tested for agglutinins specific for the strains of *V. fetus* used in the experiments (table 2).

Since the serums of all the heifers contained agglutinins against antigen 543, this strain was not employed in the experimental inoculations. Heifers whose serums showed a titer against any of the other antigens were largely excluded from inoculation with homologous cultures.

The bull used for service was a Holstein-Friesian whose serum did not contain agglutinins for Brucella or for the six strains of vibrios.

The first group of heifers (table 3) was inoculated *in utero* four to eight hours following natural service during estrus using a Folmer-Nielsen's metal catheter. This catheter is well adapted for such use, since its double spiral form permits relatively easy "threading" of the instrument through the cervix into the uterus. It has a sufficiently large opening posteriorly so that a slip-hub syringe can be attached and the material injected through the catheter. Inoculums were mixtures of approximately equal amounts of four or five of the following strains: R_5, M_5, M_2, P_1, and D_{71}.

The second group of 6 pregnant heifers (table 5) was subjected to the following inoculation schedule. Two heifers were fed 10.0 ml. of mixed Vibrio cultures in a No. 10 gelatin capsule; 2 were injected intravenously and 2 were inoculated into the uterus through a laparotomy incision.

Blood samples were taken from the heifers one week following inoculation and then at approximately two-week intervals. Tube-agglutination tests were performed utilizing the six antigens.

The heifers were killed at varying times following inoculation and the entire reproductive tract removed as quickly as possible for transport to the laboratory for studies of the bacteriology and pathology. Studies of the bacteriology were restricted to efforts to isolate vibrios. Tissues for studies of the histopathology were fixed in Mossman's fixative. The gross as well as microscopic pathological changes were studied.

The following procedures were employed in an attempt to obtain vibrios in pure culture. Fetal stomach or uterine fluids were aspirated aseptically and cultured directly in sterile semisolid Brucella broth medium and larger aliquots were placed in 30.0-ml. sterile centrifuge tubes. The tubes were centrifuged at 3,000 r.p.m. in an International centrifuge, size 1, for 20 minutes. The clear supernatant was filtered through a sterile Berkfeld W filter attached to a sterile 200.0-ml. side-arm flask. Usually, after 20 to 30 minutes, 5.0 to 10.0 ml. of filtrate could be collected. Of

TABLE 3—Group 1, Heifers Inoculated Intrauterinely with Mixed Suspensions of Vibrio Fetus Utilizing the Folmer-Nielsen's Catheter

Heifer No.	No. of inoculations	Postinoculation time killed	No. of services
697	1	62 hours	1
684	1	7 days	1
31312	3*	11 days	2
31313	5†	16 days	5
989	1	.....	4
47824	1	.....	2
89030	1	.....	4
34471	1	.....	2
694	1	.....	5
51712	1	.....	1

* Inoculated twice during the luteal phase, on days 6 and 9 following second estrus, this animal failed to conceive at first service after inoculation with *V. fetus* suspensions.

† Inoculated with mixed suspensions of *V. fetus* at each of five separate estrous periods, this heifer manifested the "repeat breeding" syndrome.

this filtrate, 1.0 ml. was pipetted into sterile semi-solid Brucella broth medium. The inoculated tubes were incubated in an atmosphere of 10.0 per cent CO_2 at 37 C. for 48 hours. With these techniques, Vibrio cultures could be occasionally isolated from known infections; however, Pseudomonas sp. also passed Berkfeld W filters and frequently overgrew the vibrios.

RESULTS

The report of the bacteriological examinations and of the gross and microscopic studies of the pathology will be limited to the significant findings.

Of the 8 heifers, which were kept a sufficient period of time following inoculation, 7 required additional service. However, 8 uninoculated control heifers became pregnant on first service. Animals were placed in an inoculated or a noninoculated group at random. Six of the 8 uninoculated control heifers (53, 691, 132, 31315, 2, and 89002) were used for additional experimentation to be defined later.

Heifer 697.—Forms resembling *V. fetus* were observed in hanging drop preparations of secretions obtained from the external and internal os of the cervix. Attempts to isolate the vibrios in culture mediums were unsuccessful because of overgrowth by molds.

Grossly, the vaginal mucosa was markedly congested and contained hemorrhagic areas measuring 3.0 to 5.0 cm. in diameter. The dorsal serosal surface of the body of the uterus was congested and ventrally there was a serous exudate, measuring 4.0 by 3.0 by 7.0 cm. Considerable pressure was required to force saline through the lumen of the right oviduct, the ovulatory side. Finally, a core of purulent material was dislodged (fig. 1) and the saline flowed freely through the oviduct.

Microscopically, the uterus displayed considerable subepithelial edema (fig. 2) and many endometrial glands contained amorphous material. A subserosal abscess was present on the left oviduct anterior to its attachment to the uterine horn. There were a number of polymorphonuclear leukocyte accumulations extending to the mesosalpinx. The right oviduct contained a core of purulent material which virtually occluded the lumen, even though a part had been washed out.

The condition in this animal was an acute bilateral salpingitis.

Heifer 684.—Hanging drop preparations of secretions from the external os and the anterior vaginal region revealed forms identical with *V. fetus*. Other regions of the genital tract were apparently free of such bacteria. Attempts to isolate vibrios by cultural methods were unsuccessful.

Grossly, suffusion-type hemorrhages were observed dorsally in the vagina and ventrally between the vagina and bladder which extended to the external urinary meatus. Vaginitis and cervicitis were observed; the cervicitis was most marked in the region of the external cervical os. The dorsal and ventral serosal surfaces of the uterus were congested. Between the body of the uterus and the bladder, extensive serous exudate was apparent over an area approximating 4.0 by 6.0 by 6.0 cm. The serosal surface of the bladder was thickened and hemorrhagic.

Microscopically, the cellular exudate of the cervix consisted mainly of plasma cells, lymphocytes, and a few polymorphonuclear leukocytes. A few of the superficial mucous cells of the cervix were necrotic and hyalinization of areas of smooth musculature was apparent. The myometrium contained a number of hyalinized areas, the interglandular areas were edematous, and numerous mast cells were present but lymphocytes predominated. The oviducts were apparently functionally normal, although there were some subserosal degenerative changes. The bladder was edematous. The vessels of the submucosa of the bladder were engorged.

This animal was afflicted with a chronic cervicitis.

Heifer 31312.—Attempts to cultivate vibrios were unsuccessful, even though many parts of the reproductive tract were cultured.

Gross pathological examinations revealed that the body and horns of the uterus were congested; an edematous exudate covered the serosal uterine surface, retroperitoneally. The dorsal serosal surface of the uterus contained a fibrinous hemorrhagic exudate which extended from the cervix to the uterine horns. The dorsal subserosa of the left uterine horn contained small abscesses 1.0 mm. to 2.0 mm. in diameter. Both oviducts contained purulent exudate.

Microscopically, the vessels of the bladder were generally engorged. The surface epithelium of the cervical invaginations

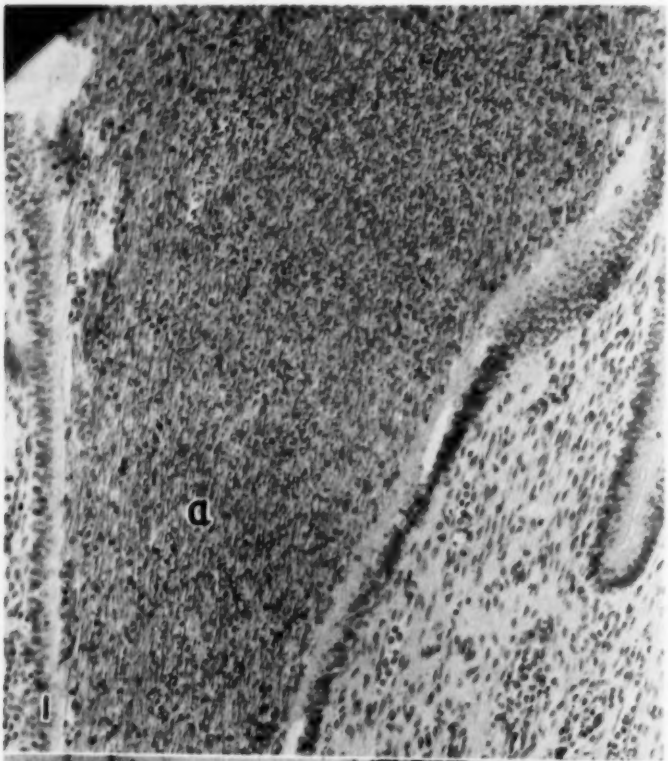

Fig. 1—Cross section of the oviduct of heifer 697, showing necrotic material in the lumen (cone-shaped portion—a), polymorphonuclear leukocytes in stroma, and salpingitis. x 165.

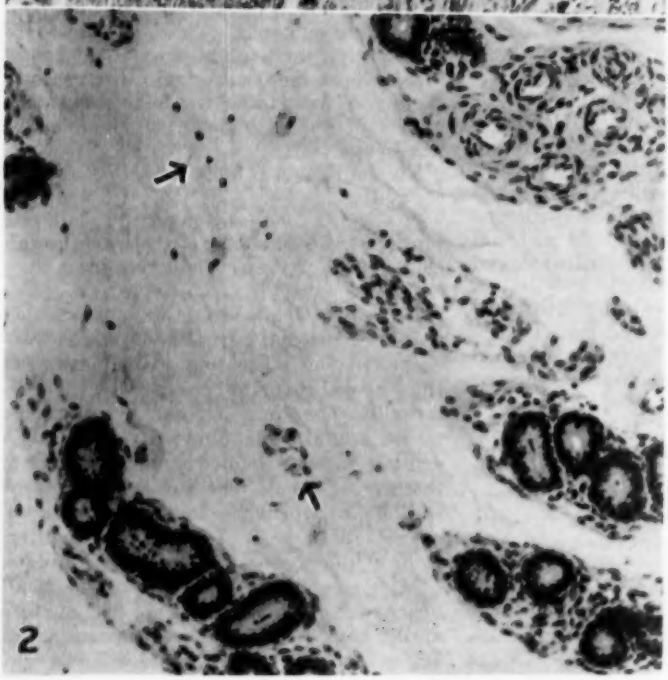

Fig. 2—Cross section of the interglandular area of the endometrium of heifer 697, showing extensive edema and nests of mast cells indicated by arrows. x 165.

Fig. 3—Cross section showing infoldings of cervical mucosa in heifer 31312 with a mild chronic cervicitis. x 165.

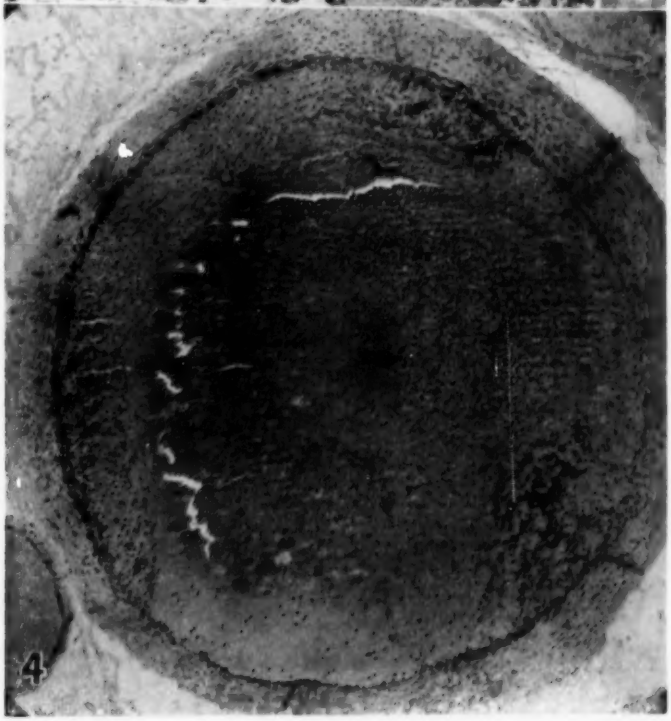

Fig. 4—Cross section of septic thrombus in a villus of heifer 53; vibrionic abortion. x 165.

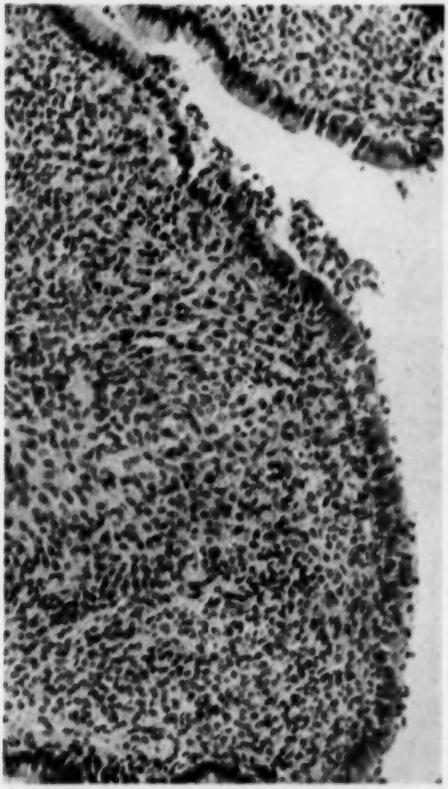

Fig. 5—Cross section of endometrium of heifer 53, showing inflammatory exudate in the subepithelium and the uterine lumen; endometritis, vibrionic abortion. x 165.

was thin and, in some areas, it was eroded and necrotic. The subepithelium contained an inflammatory exudate composed of lymphocytes, monocytes, and plasma cells. The lumen of the uterus contained an amorphous material composed largely of cellular detritus. The subepithelial endometrium contained accumulations of polymorphonuclear leukocytes and mast cells, the vessels were engorged, and a number of hemorrhagic and edematous areas were present. Numerous microscopic abscesses were observed dorsally in the mesosalpinx and the subserosa of both oviducts. The cellular exudate present within the subserosa consisted mainly of plasma cells, polymorphonuclear leukocytes, and lymphocytes.

This animal had an acute bilateral salpingitis and a chronic cervicitis (fig. 3).

Heifer 31313.—Cultures and hanging drop preparations of various parts of the reproductive tract did not reveal the presence of vibrios.

Gross pathological examination revealed a number of cysts of the vaginal mucosa which measured 0.5 to 3.0 cm. in diameter. The remainder of the reproductive tract appeared normal.

Microscopically, the surface epithelium of the cervix appeared flattened. There was vacuolization of the subepithelial tissue and a cellular exudate composed mainly of lymphocytes and plasma cells.

Considerable edema and a fibrinous exudate were observed in the subepithelium of the uterus. A cellular exudate within the endometrium consisted mainly of lymphocytes associated with a large number of mast cells. In one area of the myometrium, apparently aberrant endometrial glands were present, evidence of an endometriosis. The oviducts seemed to be normal.

This heifer had a chronic cervicitis.

The summary of the bacteriological and pathological findings of the killed heifers of group 1 (table 3) is shown (table 4).

Heifers 89002 and 2 received 5.0 ml. each of the mixed culture intravenously on alternate days until a total of five inoculations were given.

Heifer 89002 exhibited shivering, chills, and labored breathing, approximately 30 minutes following the fourth injection.

TABLE 4—Bacteriological and Pathological Findings in Heifers Inoculated Intrauterinely via Catheter

Heifer	Vibrio viewed under hanging drop preparation or recovered	Pathological changes			
		Vagina	Cervix	Uterus	Oviducts
697	+	Congested, hemorrhagic		Edema	Bilateral salpingitis
684	+	Congested, hemorrhagic	Chronic cervicitis		
31312	—	—	Chronic cervicitis	Serosal abscess	Bilateral acute salpingitis, serosal abscesses
31313	—	Cysts	Chronic cervicitis	Endometriosis	

Adrenalin® hydrochloride, 1.5 ml. of a 1:1,000 solution, was administered intramuscularly and the signs abated. She was killed 39 days after the initial inoculation. Attempts to isolate vibrios from the uterus and fetal organs were unsuccessful.

Grossly, the fetal membrane and uterus were normal in appearance.

Microscopically, two small necrotic foci were observed in the chorionic villi. A slight cellular exudation of polymorphonuclear leukocytes was seen in close proximity to the necrotic foci. The uterus was apparently normal. The tissue changes were minimal.

Heifer 2.—This heifer was killed 88 days after the initial inoculation and seven days after she had calved prematurely. The small, weak calf was killed for cultural studies. The stomach fluids were opaque and grayish. Exudate or transudate was not detected in any of the body cavities.

Organisms resembling vibrios were observed in hanging drop preparations made from the fetal stomach contents, and a pure culture of Vibrio was obtained. This heifer did not shed the tightly adherent fetal membrane and manual attempts to remove it 24 and 48 hours following the premature calving were unsuccessful. Since the uterus was ballooned and contained approximately 3 to 4 gal. of necrotic, putrid, decomposing membrane tissue and detritus, no attempt was made to culture vibrios from it.

Gross examination revealed that the fetal membrane was markedly necrotic, putrid, and unsuitable for processing and sectioning; hence, it was discarded.

Microscopically, the epithelium of the uterus in some areas was entirely eroded. The subepithelium of the uterus was infiltrated by an exudation of vast numbers of plasma cells (fig. 10), many lymphocytes, and a few polymorphonuclear leukocytes. The caruncles contained necrotic areas superficially and many of the vessels of the

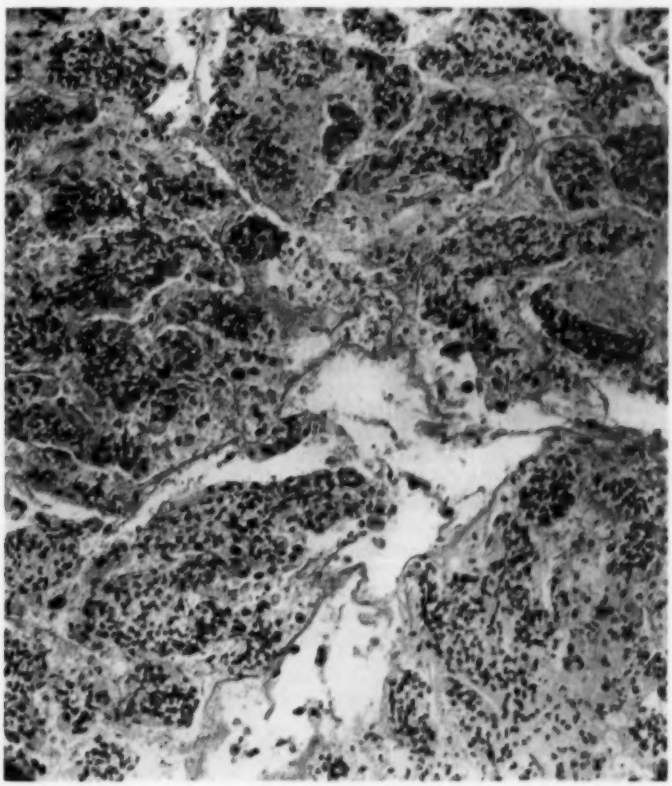

Fig. 6—Cross section of a number of necrotic chorionic villi in heifer 53; vibrionic abortion. x 165.

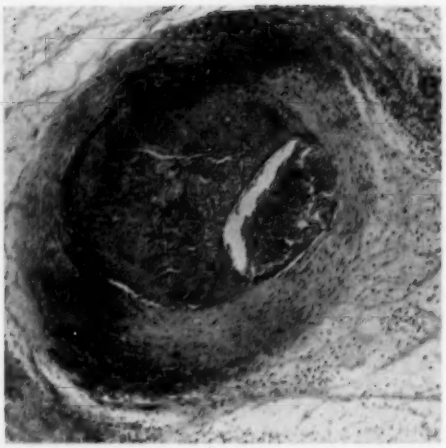

Fig. 7—Cross section of thrombosed vessel in the fetal membrane of heifer 31315; vibrionic abortion. x 165.

caruncles were thrombosed. This heifer had a severe, extensive, chronic endometritis.

The interstitial areas of the folds of the oviducts contained foci of lymphocytes and plasma cells. Although the ducts were patent, a bilateral, chronic salpingitis was present.

Heifers 691 and 13Z each received a series of five 5.0-ml. doses of mixed cultures orally. The inoculums were put into No. 10 gelatin capsules and administered with a balling gun. These cattle did not exhibit any unusual effects following administration of the capsules.

Heifer 691.—Heifer 691 was killed 39 days after initial oral inoculation. Attempts to isolate vibrionic organisms from the uterus and fetal organs were unsuccessful.

Grossly, the part of the fetal membrane which extended into the nonpregnant left uterine horn contained an edematous area approximately 3.0 by 4.0 cm. in size. The body of the uterus appeared normal.

Microscopically, many of the chorionic villi were edematous and a number appeared hyalinized. In general, the fetal tissue showed little change and the uterus was apparently normal.

Heifer 13Z.—Because the genital tract of heifer 691 was essentially normal, heifer 13Z was not killed. She was retained and calved normally. Attempts to isolate vibrios from the uterus and uterine fluids following parturition were unsuccessful.

Heifer 31315.—This heifer aborted 29 days after inoculation and was killed four days later.

The 7-month fetus was approximately normal in size for that stage of gestation. Its thoracic and peritoneal cavities each contained nearly a liter of serosanguineous fluid. Amorphous masses of gelatinous, edematous material were observed, particularly in the sublumbar region.

Vibrios were observed in hanging drop preparations obtained from the fetal stomach and the uterine fluids. These fluids were cultured and yielded growth of vibrios.

Grossly, the fetal membrane was extremely edematous, primarily in the pericotyledonary areas, while thickening and minimal necrosis were observed in the intercaruncular areas, and extensive necrotic

TABLE 5—Experimental Data of Group 2 Heifers* Inoculated Intravenously, Orally, and Intrauterinely via Laparotomy with Mixed Suspensions of Vibrio Fetus

Heifer	No. of inoculations	Postinoculation day killed	Route of exposure of *V. fetus*
89002	5	39	Intravenously
2	5	88	Intravenously
691	5	39	Orally
13Z	5		Orally
31315	1	33	Intrauterinely
53	1	33	Intrauterinely

* All the heifers of this group were bred naturally and were approximately in the fifth month of pregnancy at the time of exposure.

TABLE 6—Bacteriological and Pathological Findings in Pregnant Heifers Inoculated Intravenously, Orally, or Intrauterinely via Laparotomy

Heifer	Vibrio recovered or viewed under hanging drop preparation	Pathological changes			
		Vagina	Cervix	Uterus	Oviduct
89002	—	—	—	—	—
2	+	—	—	Chronic endometritis	Bilateral chronic salpingitis
691	—	—	—	—	—
13Z	(No necropsy)				
31315	+	—	—	Acute endometritis	Acute bilateral salpingitis
53	+	—	—	Acute endometritis	—

foci were observed in the cotyledonary attachments. The fetal membrane was heavy as a result of extensive generalized edema. The uterus contained 3 liters of serosanguineous fluid with necrotic debris. The uterine wall appeared somewhat thickened. The endometrium seemed congested and the caruncular areas were generally necrotic.

TABLE 7 — Reciprocal Tube-Agglutination Test Results—Heifer 51712*

Date	Antigens					
	543	R_2	M_2	M_3	D_{71}	P_1
3/1	320	80	10	160	20	—
3/5	320	80	80	160	80	—
3/19	320	320	160	320	80	160
4/3	320	320	40	80	40	160
4/17	160	160	20	160	80	160
5/1	160	160	80	160	80	160
5/15	160	160	80	160	80	160
6/5	320	320	80	320	160	320
6/19	320	320	320	640	160	320

* Bred Feb. 28, 1951, and inoculated intrauterinely Feb. 28, 1951, with *V. fetus*, strains R_2, M_2, M_3, and D_{71}.

Microscopically, the fetal membrane exhibited an inflammatory edema associated with infiltration of polymorphonuclear leukocytes and a few lymphocytes and plasma cells. The vessels of the villi contained septic thrombi (fig. 7). The lumen of the oviducts contained eosinophilic amorphous debris. Accumulations of polymorphonuclear leukocytes were seen in the interstitial areas of the submucosa as well as occasional foci of chronic inflammatory cells (fig. 9). The caruncles contained necrotic areas (fig. 8) and, in some fields, there were adhesions between the chorionic villi and the spaces within the caruncles in which the chorionic villi interdigitate. The interglandular areas contained numerous edematous areas.

This animal had an acute endometritis with a bilateral acute salpingitis.

Heifer 53.—This heifer calved prematurely 22 days after inoculation and was killed 11 days later.

The calf was normal in size and appearance for one at 6 months' gestation. It was killed for bacteriological study. Hanging drop preparations of the fetal stomach contents and the uterine secretions revealed numerous vibrionic forms. The stomach content yielded a pure culture of Vibrio organisms. A yellow, amorphous debris with a viscosity similar to that of meconium was present in the stomach.

The edematous and thickened fetal membrane was expelled *in toto* approximately six hours following parturition. The caruncular attachments were necrotic, as in heifer 31315, and the pericotyledonary areas were conspicuous because of their large, thick, edematous condition. In some areas, the fetal membrane was leathery, and markedly necrotic, with some foci 6.0 cm. in diameter. Between the tissue layers in many areas, hemorrhagic exudate ranging from 1.0 to 5.0 cm. in diameter was observed. The uterus contained amorphous necrotic debris in the lochia. In general, the endometrium of the pregnant horn was hemorrhagic, that of the nonpregnant horn somewhat congested.

Microscopically, most of the chorionic villi were necrotic (fig. 6) or markedly degenerated. Possibly, the most significant finding was the extensive inflammatory edema between the layers of the fetal membrane. The exudate consisted primarily of polymorphonuclear leukocytes with some lymphocytes and plasma cells. Many of the vessels of the villi were thrombosed (fig. 4) and acute and chronic inflammatory cells were observed in most areas. The lumen of the uterus contained an exudate

TABLE 8—Reciprocal Tube-Agglutination Test Results—Heifer 2*

Date	Antigens						Brucella
	543	P_1	M_3	D_{71}	R_2	M_2	
4/19	160	160	320	160	160	160	—
4/21	160	160	160	80	160	80	±
4/23	320	320	320	160	320	160	±
5/1	1,280	1,280	1,280	40	1,280	1,280	+++I
5/9	2,560	1,280	5,120	80	640	320	+I
5/17	2,560	1,280	5,120	320	1,280	160	+++—
5/22	2,500	1,280	2,560	640	320	160	+++
5/31	640	320	1,280	320	320	160	+—
6/5	640	320	1,280	320	640	80	+—
6/19	160	160	1,280	320	640	160	—

* Bred Nov. 16, 1950, and inoculated intravenously April 15, 17, 19, 21, and 23, 1951, with *V. fetus*, strains P_1, M_3, D_{71}, R_2, and M_2.

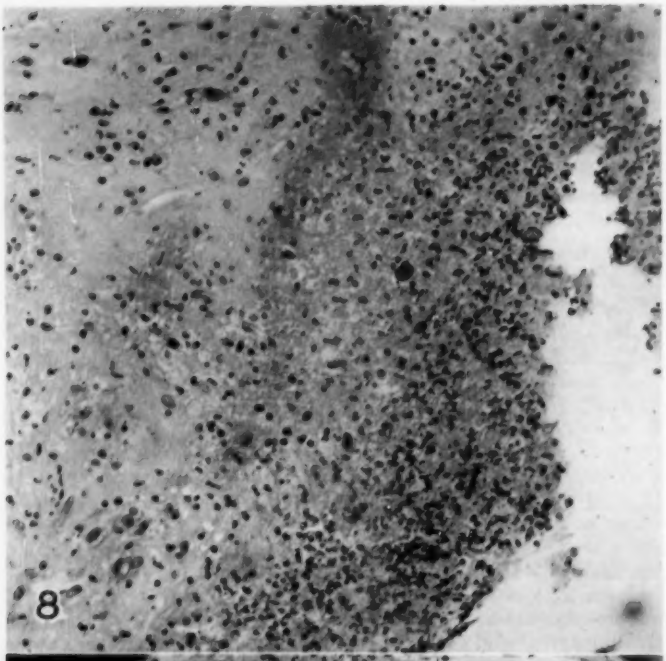

Fig. 8—Cross section area of focal necrosis, showing uterine caruncle of heifer 31315; vibrionic abortion. x 165.

Fig. 9—Cross section of an endometrial gland in heifer 31315, showing extensive exudate consisting predominantly of polymorphonuclear leukocytes; vibrionic abortion. x 165.

TABLE 9—Reciprocal Tube-Agglutination Test Results—Heifer 89002*

Date	Antigens						Brucella
	543	P_1	M_3	D_{71}	R_3	M_2	
4/19	320	160	320	80	160	40	—
4/21	320	320	320	80	80	80	—
4/23	320	320	640	320	320	80	—
5/1	640	320	640	80	640	80	++I
5/9	320	320	320	80	320	80	++—
5/17	1,280	1,280	640	160	1,280	160	+++I
5/22	2,560	1,280	2,560	320	2,560	320	+++—
5/24†	2,560	1,280	2,560	80	320	160	++I

* Bred Nov. 15, 1950, and inoculated intravenously April 15, 17, 19, 21, and 23, 1951, with *V. fetus*, strains P_1, M_3, D_{71}, R_3, and M_2.
† Killed.

composed chiefly of polymorphonuclear leukocytes and lymphocytes; plasma cells were also present (fig. 5). The vessels of the endometrium were engorged and mast cells were present in great numbers in the endometrium and myometrium. The inflammatory exudate of the endometrium consisted largely of polymorphonuclear leukocytes. Sections of the caruncles revealed areas of necrosis and hyalinization of the more superficial regions.

The major pathological changes in this heifer consisted of an acute endometritis accentuated by extensive edema.

The summary of the bacteriological and pathological findings of the heifers of group 2 (table 5) is shown (table 6).

The serum of each heifer was tested periodically for agglutinins for *V. fetus*. Representative titers are compiled (tables 7 to 12). Marked differences in the titers existed among the heifers and the reactions of individual serums with the six antigens varied considerably from time to time. The serums from 2 animals which were inoculated intravenously contained the highest titer of agglutinins for the Vibrio antigens. The titers of 2 heifers, inoculated intrauterinely via laparotomy, which aborted 22 and 29 days later, respectively, were somewhat higher for antigens M_3 and R_3. Reactions with the other four antigens did not change appreciably. The serums of the 2 heifers which received mixed cultures of Vibrio organisms orally agglutinated the antigens only in low dilutions. However, there was some increase in agglutinins for the antigens M_3 and R_3. Animals exposed to all five strains showed a greater response to R_3 and M_3.

A significant finding was that agglutinins for Brucella developed in 2 heifers inoculated intravenously with *V. fetus*. The Brucella titer reached a maximum approximately one month after the initial inoculation and rather rapidly subsided thereafter. Heifer 89002 was slaughtered at 39 days when her titer was receding,

TABLE 11—Reciprocal Tube-Agglutination Test Results—Heifer 32788*

Date	Antigens					
	543	R_3	M_2	M_3	D_{71}	P_1
2/26	160	80	40	80	80	—
3/5	160	80	40	80	80	—
3/19	160	80	40	80	80	80
4/3	320	40	20	160	80	160
4/17	320	40	80	160	160	80
5/1	320	80	80	160	40	320
5/15	320	320	40	320	40	160
6/5	640	320	80	640	320	320
6/19	320	320	80	640	160	160

* Bred Feb. 23, 1951, and March 20, 1951 (vaginatore), and inoculated intrauterinely via catheter March 20, 1951, with *V. fetus*, strains D_{71} and M_3.

TABLE 10—Reciprocal Tube-Agglutination Test Results—Heifer 691*

Date	Antigens						Brucella
	543	P_1	M_3	D_{71}	R_3	M_2	
4/19	320	160	160	160	160	80	—
4/21	160	160	160	40	80	40	—
4/23	320	160	320	160	40	80	—
5/1	160	160	160	40	80	80	—
5/9	160	160	160	40	80	40	—
5/17	320	160	320	80	320	20	—
5/22	320	320	640	160	640	80	—
5/24†	320	160	640	160	160	80	—

* Bred Nov. 12, 1950, and inoculated orally April 15, 17, 19, 21, and 23, 1951, with *V. fetus*, strains P_1, M_3, D_{71}, R_3, and M_2.
† Killed.

TABLE 12—Reciprocal Tube-Agglutination Test Results—Heifer 13Z*

Date	Antigens						
	543	P_1	M_2	D_{75}	R_3	M_5	Brucella
4/19	160	160	160	20	80	80	—
4/21	160	160	160	20	160	80	—
4/23	160	160	160	20	80	80	—
5/1	320	160	320	20	80	40	—
5/9	320	160	320	80	160	20	—
5/17	320	160	640	80	320	160	—
5/22	320	320	1,280	320	640	80	—
5/31	640	320	2,560	320	320	160	—
6/5	320	320	1,280	320	320	160	—

* Bred Nov. 20, 1950, and inoculated orally April 15, 17, 19, 21, and 23, 1951, with *V. fetus*, strains P_1, M_2, M_5, R_3, and D_{75}.

while heifer 2 did not possess a Brucella titer two months following the initial Vibrio inoculation. The maximum titer reached was 1:100, with incomplete agglutination at the 1:200 dilution. The Brucella titer manifested in these heifers is considered to be the result of an antigen common to Vibrio and Brucella. No evidence of brucellosis was found in any of the animals at any time.

The results of a limited number of trials in which the cervical mucus was tested for agglutinins were equivocal. The uterine fluid titers of the 2 heifers which had been inoculated intrauterinely via laparotomy were of the same magnitude as the blood titers.

DISCUSSION

The pathological alterations which could be ascribed to Vibrio inoculations were de-

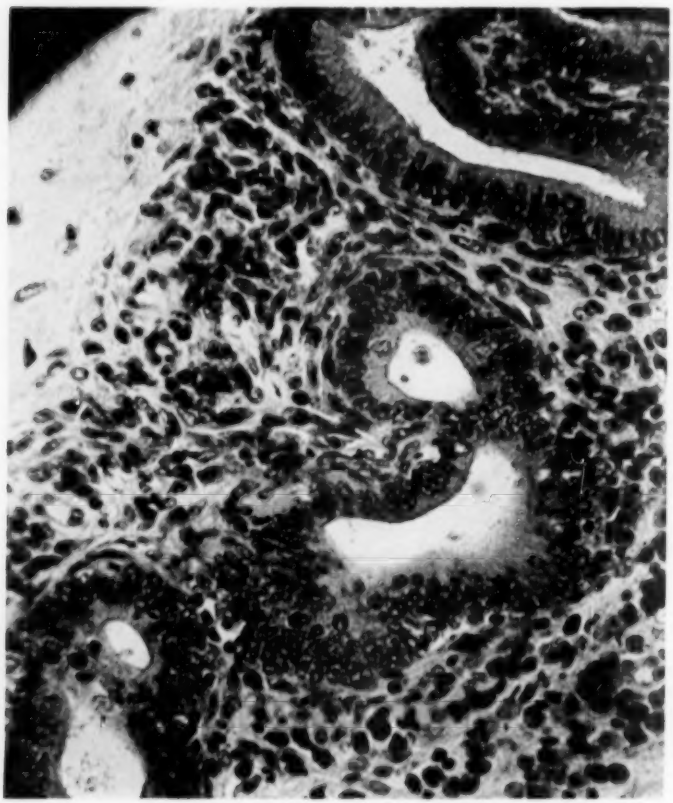

Fig. 10—Cross section of an interglandular area of endometrium of heifer 2; exudate predominantly of plasma cells; chronic endometritis, vibrionic abortion. x 330.

pendent somewhat upon the route of inoculation, stage of estrous cycle, and stage of pregnancy. The most significant change was an acute salpingitis in heifer 697. It is believed that at estrus the uterus is conditioned physiologically so that semen can be propelled into the oviduct. It is reasonable that foreign material introduced into the uterus at estrus would likewise be propelled into the oviduct. On the basis of this theory, it can be surmised that the foreign material in the form of Vibrio suspensions could by mechanical or by inflammatory incitement prevent conception. The fact that 1 heifer conceived following inoculation with vibrios would tend to discredit this theory although sufficient disturbance of the oviduct may not occur to prevent conception.

The pathological alterations af the uterus were most marked in the heifers which aborted subsequent to intrauterine inoculation via laparotomy and in heifer 2 inoculated intravenously.

The uterine and fetal membrane alterations were minimal in 2 heifers which were slaughtered approximately one month following oral and intravenous inoculations, respectively, of Vibrio cultures.

The uterus of heifer 31313, manifested a number of tissue changes which were indicative of a chronic endometritis. This heifer which had been rebred five times and inoculated five times was slaughtered 16 days following the last breeding. The significance of the aberrant endometrial glands in the myometrium, *i.e.*, endometriosis, is open to speculation. This focus of endometrial glands was located in the myometrium and not in the usual subepithelial location.

There was no evidence of permanent injury due to salpingitis in the limited number of animals in this experiment, nor was there any indication that hydrosalpinx followed the salpingitis. The most severe reaction was observed in the heifer inoculated twice during the luteal phase of the estrous cycle. This is additional evidence that the uterus is less able to cope with infection during the luteal phase [36] than at estrus and that endocrine influence plays a major part in the control of uterine infections.

Since the reactions of individual serums varied with the six antigens, time of exposure, and route of exposure, it is obvious that the agglutination test using serum for the routine diagnosis of vibriosis is of questionable value (tables 7 to 12).

Summary

1) Exposure of heifers to *Vibrio fetus* at the time of estrus resulted in the "repeat breeding" syndrome.

2) Cultures of *V. fetus*, inoculated intrauterinely via laparotomy and intravenously by the external jugular vein, were capable of causing premature births.

3) The major pathological changes observed in experimentally induced vibriosis were: salpingitis, endometritis, and extensive generalized inflammatory edema of the fetal membrane.

4) *Vibrio fetus* infection in apparently Brucella-free cattle resulted in the development of positive blood-agglutination titers to brucellosis.

5) The value of the tube-agglutination test using serum for the routine detection of vibriosis is equivocal.

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Paper Electrophoresis of Bovine Serum

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PAPER ELECTROPHORESIS is a simple, rapid method for the separation and subsequent identification of the components of complex ionic mixtures. The separation of serum proteins, in particular, has been extensively studied by the paper electrophoretic technique.

Scattered reports are available on the electrophoretic examination of animal serums. Vesselinovitch and Funnel[10] studied both human and canine serums by a two-hour paper electrophoretic procedure. Polson and Malherbe[5] analyzed canine serums in several disease conditions using moving boundary electrophoresis. Vesselinovitch[9] described electrophoretic studies on edema disease in swine. Chabaud *et al.*[2] used electrophoresis in the antemortem diagnosis of rabies. San Clemente and Huddleson[8] and Pierce,[4] using the moving boundary method, reported extensive studies on both normal and immune bovine serum.

The present report is preliminary in nature and intended primarily to call attention to the value of paper electrophoresis in the study of bovine serum. The greater part of the work to date has been directed to the development of a buffer, time, and current system satisfactory for routine examination of bovine serum.

METHODS

A Durrum-type, hanging strip cell which holds eight 3- by 30-cm. paper strips and a Spinco Duostat power source were used throughout these studies.* Samples of 0.006 to 0.01 ml. of serum were separated on Whatman 3-mm. filter paper strips and stained for proteins, lipoproteins, and glycoproteins according to the methods given by Block *et al.*[1] Bromphenol blue (protein) strips were scanned with the Analytrol recording scanner and integrator.*

Veronal buffers of pH 8.6, varying in ionic strength (μ) from 0.03 to 0.075, were tested under different time and current conditions. Phosphate buffers were also tested, but the patterns tended to smear unless long runs at low milliamperage were employed. Throughout this work constant current (milliamperes) rather than constant voltage was employed.

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This paper was prepared with the technical assistance of Mrs. Eugene Manus.

* Specialized Instruments Corp., Belmont, Calif.

RESULTS

Three general types of veronal buffer systems were employed: (1) long time, low current, high ionic strength; (2) short time, high current, low ionic strength; and (3) conditions intermediate between 1 and 2.

1) A 16-hour, 5-ma., $\mu = 0.075$ system, recommended for human serum,[1] gave excellent resolution of albumin and *alpha* globulin but poor resolution of *beta* and *gamma* globulins (fig. 1B).

2) Two- and three-hour runs at 30 to 35 ma. and low ionic strengths (0.03 to 0.053) produced patterns similar to system 1. Mobility of all components was greatly decreased and distortion of the protein bands was extreme.

3) Two intermediate systems gave the most satisfactory results: *(a)* eight hours, 12 ma., $\mu = 0.053$ and *(b)* five hours, 21 ma., $\mu = 0.053$. The eight-hour system was developed first and utilized most extensively. The five-hour system, however, produced comparable separations and was more practical in an eight-hour working day. With both systems, there was good separation of albumin and *alpha* globulin, but undesirable diffusion frequently occurred in eight-hour runs. There was good resolution of the *beta* and *gamma* globulins. In all eight-hour and most five-hour runs, two *gamma* globulins could be identified (fig. 1A; fig. 2A and B).

Attempts to reduce time with proportional increases in milliamperage or decreases in ionic strength resulted in band distortion and failure to separate the two *gamma* globulins.

Normal bovine serum protein patterns with the 16-, eight-, and five-hour systems are shown (fig. 1 and 2). Although insufficient samples from different breeds, ages, and sexes under varying environmental conditions are available to establish statistical values, the patterns we have observed in bovine serum have been fairly

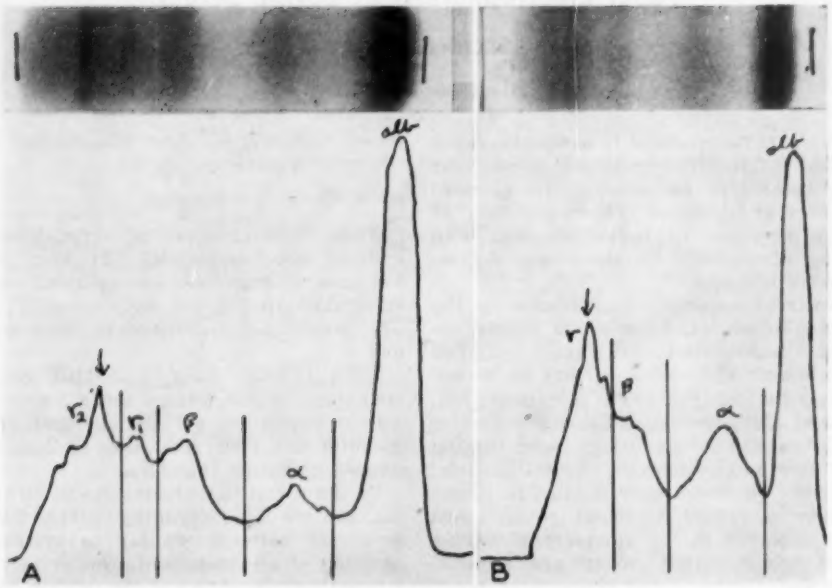

Fig. 1—(A) Normal bovine serum protein pattern and scan run eight hours, 12 ma., $\mu = 0.053$. Note the separation of two gamma globulin components.
(B) Normal bovine serum protein pattern and scan run 16 hours, 5 ma., $\mu = 0.075$. Only one gamma component is apparent.

regular. The following percentage composition of a serum sample from a normal 5-year-old Holstein-Friesian cow was calculated from a test with the five-hour system: albumin, 52 per cent; *alpha* globulin, 13 per cent; *beta* globulin, 11 per cent;

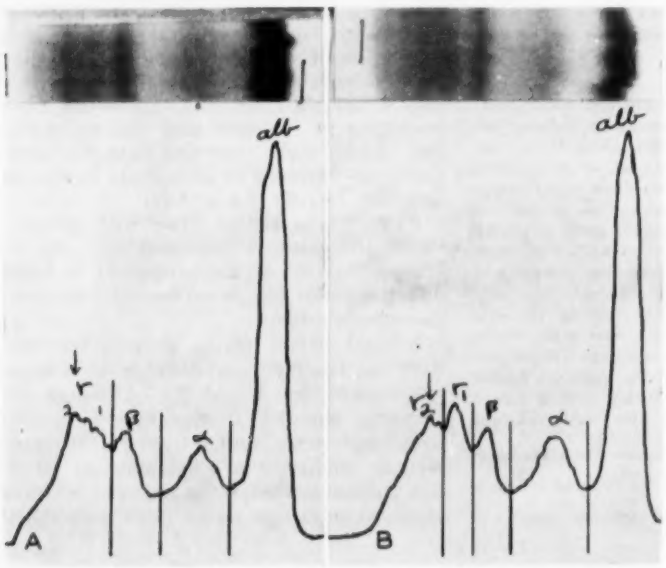

Fig. 2—(A) Normal bovine serum protein pattern and scan run five hours, 21 ma., $\mu = 0.053$. Gamma$_1$ globulin is apparent but not adequately separated.
(B) Normal bovine serum protein pattern and scan run five hours, 21 ma., $\mu = 0.053$. Adequate resolution of gamma$_1$ and gamma$_2$ globulins.

gamma$_1$ globulin, 9 per cent; and *gamma*$_2$ globulin, 15 per cent.

Studies are now under way to establish normal values and ranges for the different breeds, ages, and sexes.

Considerable variation in percentage composition was usually found when the same serum was tested by several different systems. The differences could usually be attributed either to jamming or diffusion of the albumin and *alpha* globulin bands with all except the five-hour system.

Bovine serum did not separate as well as human serum and showed more diffusion of the individual components (compare fig. 1, 2, and 3). Bovine *alpha* globulin failed to separate into two components with any system tested. Human *alpha* globulin consistently separated into *alpha*$_1$ and *alpha*$_2$ components (fig. 3). *Beta* globulin and *gamma*$_2$ globulin migrated in opposite directions and were readily identified. The *gamma*$_1$ component was difficult to interpret. This band was first considered to be a *beta*$_2$ globulin because of its migration toward the positive pole immediately behind *beta* globulin. Pierce [4] showed, with moving boundary electrophoresis, that two and often three *gamma* globulins can be detected in bovine serum. This finding, together with our observation that this component either varies or merges with *gamma* rather than *beta* globulin in some abnormal serums, led to the conclusion that it was a *gamma*-type globulin. More definitive studies are required to determine the exact nature of this component.

Fig. 3—Normal human serum protein pattern and scan run five hours, 21 ma., $\mu = 0.053$. Note alpha$_1$ and alpha$_2$ globulins.

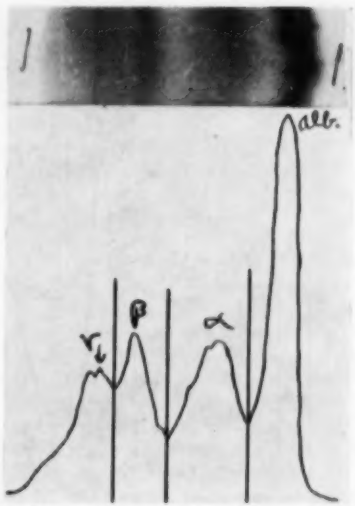

Fig. 4—Ramsey-type mucosal disease. Notice the increase in alpha globulin as compared to the normal.

Glycoproteins and lipoproteins were studied to only a limited extent. Schiff-staining glycoproteins were demonstrated to be migrating with albumin, *alpha*, *beta*, and *gamma*$_2$ globulin. Three lipoprotein components were detected with the oil red O stain; one migrated between albumin and *alpha* globulin, another with *beta* globulin, and the third remained at its origin. Separation of lipoproteins was erratic and was accomplished to any appreciable extent only with the 16-hour, 5 ma., $\mu = 0.075$ system. Five- and eight-hour runs have produced only smearing and a rare trace of separation. More work is required to establish a satisfactory method for resolving bovine serum lipoproteins.

Seven abnormal bovine serum patterns prepared by the five-hour system are shown (fig. 4 to 8). The serum pattern of an animal with a case of Ramsey-type mucosal disease is shown (fig. 4). The *alpha*

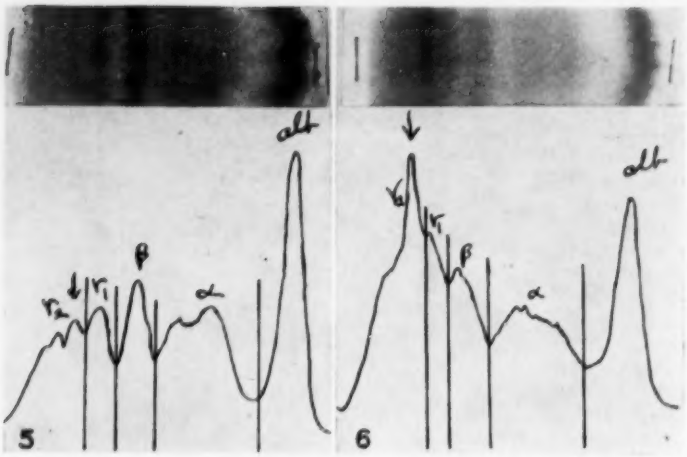

Fig. 5—Lymphomatosis. There is an overall increase in globulins, particularly gamma globulin, and a marked decrease in albumin.

Fig. 6—Streptococcic endocarditis, fibrosis and chronic passive congestion of the liver, and chronic interstitial nephritis. Notice the depressed albumin and increased alpha and gamma globulins.

globulin is increased about twofold (26%). *Alpha* globulinemia is associated with inflammation and tissue destruction.[3] Oral, esophageal, and abomasal ulceration as well as hemorrhagic enteritis and colitis were found at necropsy. The serum pattern of an animal with a case of lymphomatosis (lymphosarcoma), and the only example of a double *alpha* globulin peak encountered in this work, is shown (fig. 5). There is marked depression of albumin (25%) and concomitant increase in globulins, particularly *gamma* globulin (30%). Prior to necropsy, three hematological examinations failed to reveal any increase in numbers of lymphocytes or any evidence of abnormal lymphocyte morphology. The serum pattern for a cow with tricuspid vegetative endocarditis is shown (fig. 6). The albumin is depressed (23%), and

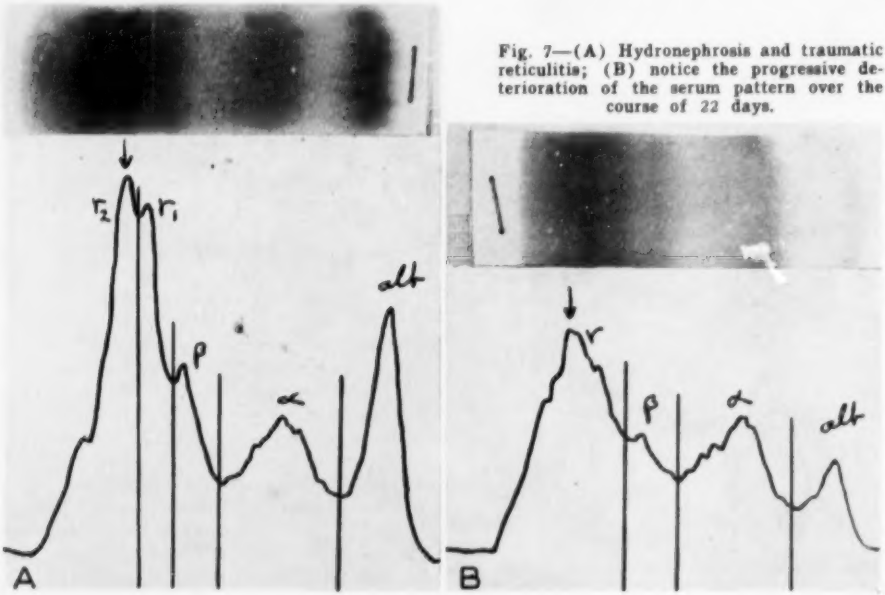

Fig. 7—(A) Hydronephrosis and traumatic reticulitis; (B) notice the progressive deterioration of the serum pattern over the course of 22 days.

alpha globulin (26%) and *gamma* globulins (38%) increased. In addition to the endocarditis, there was chronic passive congestion and diffuse fibrosis of the liver, as well as chronic interstitial nephritis. Both renal albumin loss and suppression of albumin synthesis by the liver could account for the low albumin and relative increase of globulins observed.[6] The hypergammaglobulinemia may, also, reflect an immune reaction to the hemolytic Streptococcus isolated from the heart lesion.

Figures 7 A and B are from a case of traumatic reticulitis and hydronephrosis in a Guernsey heifer. The initial diagnoses were traumatic gastritis and an unspecified enlargement of the left kidney. A rumenotomy was performed and three nails removed from the reticulum. The animal failed to recover satisfactorily and a necropsy was done 22 days later. Severe left hydronephrosis and multiple infarction of the right kidney as well as reticulodiaphragmatic adhesions and abscesses were found. The ventral third of the liver was fixed to the diaphragm by multiple adhesions. Serum A was collected at the time of operation and shows depressed albumin (17%) and elevated *gamma* globulins (49%). Sample B was taken prior to necropsy 22 days later. The nephrosis had progressed as reflected in a further depression of albumin (7%). *Alpha* globulin had increased from 19 per cent to 29 per cent, while the *gamma* globulins remained the same. In man, a low albumin, high *alpha* globulin, and low *gamma* globulin pattern is considered characteristic of nephrosis,[6] while a normal *alpha* globulin in the presence of low albumin and high *gamma* globulin is a reasonably constant finding in liver disease.[3] The present case appears to represent a mixture of these two pictures, reflecting the presence of both liver and renal disease. A similar electrophoretic pattern was observed in a case of bovine amyloidosis with involvement of both liver and kidney.[7]

Figures 8 A and B represent serums from a Shorthorn heifer with a disease like fescue foot disease. Sample A was tested during the acute febrile period with severe lameness and inflammation in the hindfeet. Albumin is only 24 per cent, while *gamma*

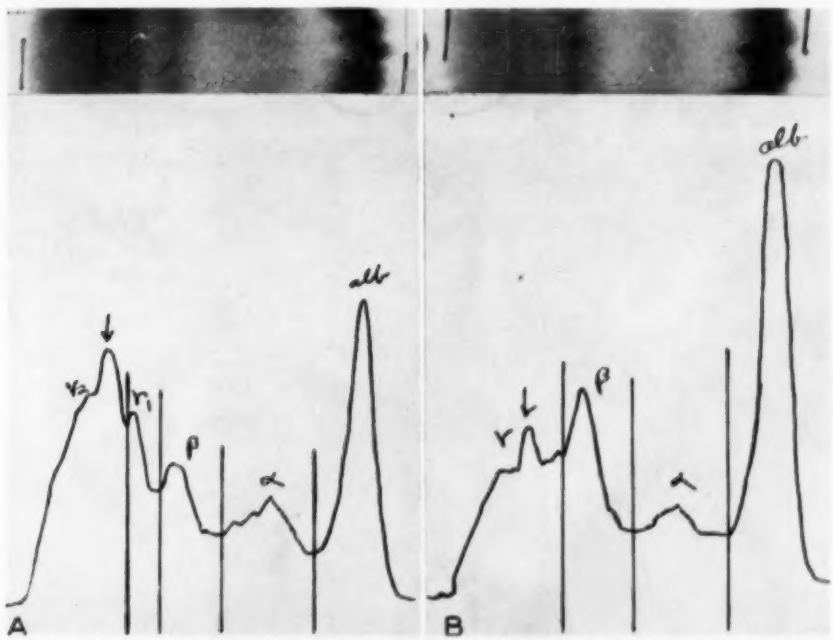

Fig. 8—A disease like fescue foot. The "liver disease" pattern (A) has virtually returned to normal (B) five months after the acute phase of the disease.

globulins are 48 per cent. The normal *alpha* globulin (14%) together with low albumin and high *gamma* globulin is reasonably characteristic of liver disease in man.[3] The electrophoretic pattern (B) had virtually returned to normal five months later when the animal was slaughtered because of chronic deformities of the hindfeet. No gross or microscopic lesions could be demonstrated in the liver at that time.

SUMMARY

A system for the paper electrophoretic analysis of bovine serum is presented. A five-hour run at 21 ma. constant current, using a $\mu = 0.053$ veronal buffer system is recommended. The hanging strip cell and Whatman 3-mm. filter paper were used. Examples of abnormal bovine serum protein patterns are discussed. Paper electrophoresis appears to be a valuable adjunct to the study of pathological physiology in cattle.

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A Rumen Fistula Modification

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THE RUMEN fistula is a valuable aid in teaching, research, and even in practice.

Since the early work of Schalk and Amadon,[1] it has been used in many ways, such as in the relief of chronic bloat and in the study of traumatic gastritis (Dougherty [2]). Nangeroni [3] reported a fistula that had been functioning satisfactorily for 12 years. Michael and McKinley [4] simplified the classic operative technique.

The fistula to be described, using a circular incision, has been established satisfactorily in 12 attempts in 13 adult dairy cattle in the last seven years. Two pairs of identical twins were in the group. The one unsuccessful operation will be discussed later.

Fig. 1—A circular incision has been made in the rumen wall ¾ of an inch from the continuous sutures for approximately one half of the circumference only, and several retention sutures (mattress type) are inserted. The excision and retention suturing are then completed.

THE OPERATION

The size of the fistula is limited by the paralumbar fossa (Sisson [5]). With an indelible pencil, a circular mark is made to indicate the line of incision. Adequate anesthesia is provided by the 2 per cent procaine infiltration described by Frank.[6] The skin incision is made with a hooked knife (Bard-Parker No. 12), and the skin is ex-

Dr. Schnautz is from the Oregon Agricultural Experiment Station, Corvallis.

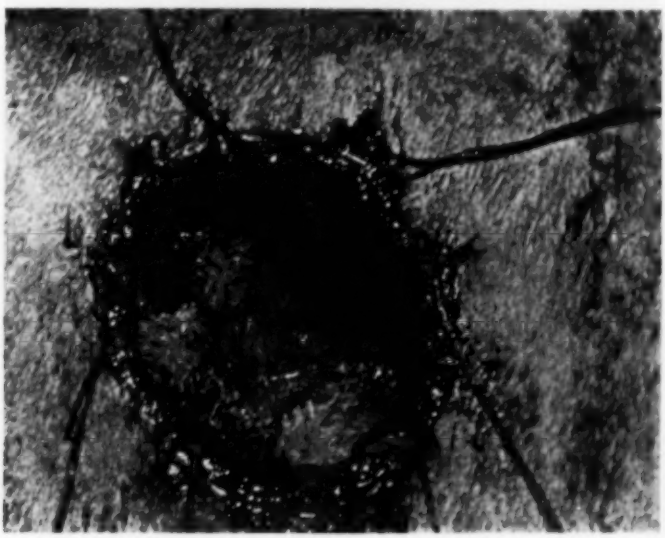

Fig. 2—The fistula is completed but the umbilical tape ties, held by assistants during suturing, are still attached.

Fig. 3—The completed fistula, with plug and transparent cover in place. The lower retention sutures are showing.

cised. A similar portion of the flank muscles and peritoneum is then removed.

The rumen wall is then grasped with a cervical forceps and drawn into the opening. It is kept in position by inserting umbilical tape at four points, to be held by assistants or anchored to the skin. A continuous circle of No. 4 chromic gut suture is used to attach the rumen to the skin margin. The stitches do not penetrate the mucous membrane of the rumen. After suturing, the fistula is completed by excising the circular portion of the wall of the rumen approximately $\frac{3}{8}$ of an inch from the suture line (fig. 1 and 2), making a $4\frac{1}{2}$-in. hole to accommodate the light weight plastic plug. Tape retention sutures of a through-and-through (the rumen and body wall) mattress type are employed at 2- to 3-in. intervals, some 2 in. from the margin of the fistula. These take most of the strain off the marginal sutures and insure a broad line of adhesion to the abdominal wall. They are removed after six to eight days.

A four-piece plastic, threaded plug assembly with a transparent cover is inserted and screwed together loosely, and the fistula is ready for use immediately (fig. 3). No after-care is needed. Antibiotics are used on the wound surface before suturing, and procaine penicillin is given intramuscularly.

In early trials, the rumen was sutured to the muscle and to the peritoneum, but no retention sutures were used. In the first 3 animals on which this operation was performed, only one line of sutures (rumen to skin) was used. However, in the

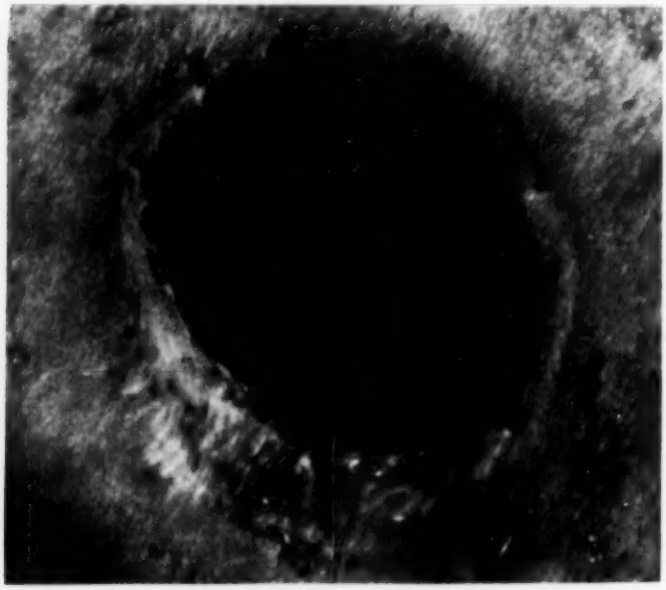

Fig. 4—The open fistula 16 days after surgery. Healing is complete.

third one, the sutures pulled out at the lower border, allowing seepage into the abdominal cavity with fatal results. Retention sutures have overcome that hazard. The exposed portion of the rumen wall should be removed immediately. When it was allowed to remain until healing had occurred, it became considerably thickened and a less satisfactory fistula margin remained. The operation can usually be completed in two hours.

This circular opening method requires less care and has less tendency to constrict than does the conventional method. Healing is rapid and uniform and the difficulties associated with healing at the wound commissures are averted. There is no distortion of the rumen wall (fig. 4) and less discomfort to the animal when inserting and removing the plug.

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Studies on Bovine Leptospirosis. III. Isolation of *Leptospira Pomona* from Surface Waters

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It appears to be conclusively established that certain leptospiral infections of man, such as Weil's disease and swamp fever, are primarily water-borne. A recent incident in Alabama[1] clearly indicates that exposure to water contaminated with *Leptospira pomona* may result in a high incidence of infection in man. More recently, another person was infected, apparently while swimming in a contaminated pond.[2] It seems reasonable that epizootics in cattle caused by this species may be attributable, at least partially, to dissemination of the agent through water.[3-5]

While studying epizootics of *L. pomona* infection in cattle, we have often succeeded in recovering the organism from surface waters. The sources from which recoveries were made include three streams in widely separated natural drainage basins and from pools on four different ranches—two in corrals and three in pastures. Four of the pools were created by overflow from stock tanks, the other by a leaking irrigation hydrant. All sources were frequented by infected cattle, some of which were known to be shedding large numbers of leptospiras in the urine.

The recovery from water samples was correlated in every instance with the presence of cattle excreting infectious urine. This correlation was established through demonstration of leptospiras by dark field examination and by concurrent inoculation of the dark field-positive urines into laboratory animals. *Leptospira pomona* was subsequently recovered in pure culture from five of the specimens. The results of repeated serological tests on a herd basis provided conclusive evidence of coincident epizootic conditions in these herds.

Experimental Methods

Water samples were collected in sterile flasks or tubes after the water was agitated sufficiently to bring some sediment to the surface. This was done because of the report of investigators in Holland[5] that the organisms reside on or near the bottom of pools and streams. In collecting water from streams, a small bay or eddy was selected where the flow was more or less retarded. As soon as possible, samples were either transported to the laboratory for animal inoculation or injected into animals in the field. The animals inoculated were weanling to half-grown hamsters and guinea pigs, and sometimes chickens, 2 to 6 weeks old. No attempt was made to concentrate specimens. The water was injected intraperitoneally in quantities considered to be as large as the animals would tolerate. During the first phase of the study, additional guinea pigs were exposed to large quantities of water by a "bathing" technique.[6] This involved immersing areas of their shaved and abraded abdominal skin in slowly flowing water siphoned from the sample container. In this way, they were exposed over a period of about two hours to approximately 250 ml. of water.

The following methods were used, in various combinations, to detect or confirm infection in the inoculated animals: *(a)* culturing or dark field examination of peritoneal fluid, *(b)* observing for febrile reactions, *(c)* blood cultures, *(d)* serological tests, *(e)* culture of tissues, and *(f)* examination of Levaditi-stained kidney sections. Identity of the organism was first indicated by the demonstration of agglutinins for *L. pomona* and the organisms were finally identified, after isolation, by agglutination tests with high-titer, type-specific antiserums. The serums used for the latter purpose were produced by immunization of rabbits with known strains* of *L. pomona*.

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Department of Veterinary Science, Washington Agricultural Experiment Stations (Gillespie and Kenzy); Department of Veterinary Microbiology, College of Veterinary Medicine (Ringen); and Department of Veterinary Clinical Medicine and Surgery, College of Veterinary Medicine (Bracken), State College of Washington, Pullman.

The authors gratefully acknowledge the cooperation of practicing veterinarians, especially Drs. P. M. Aldrich and L. L. Newman, and of regulatory officials in the Division of Dairy and Livestock, namely Drs. A. E. Crouse and C. E. Eakin, through whose interests the infected herds were located. They also express gratitude for assistance and advice offered by personnel of the U. S. Public Health Service, Rocky Mountain Laboratory at Hamilton, Mont., particularly Drs. K. R. Reinhard and H. G. Stoenner.

* Strain 812 obtained from Lederle Laboratories, Pearl River, N. Y., and strain NYB-1 from Dr. Karl Reinhard, Rocky Mountain Laboratory, Hamilton, Mont.

RESULTS

The organism was recovered from eight water samples, on five different dates, from eight separate sources (5 pools and 3 streams) on ranches in southeastern and central Washington. All provided drinking water for, and were subject to contamination by, herds in which infection was known to exist. The presence of infection in the herds was first indicated by official serodiagnostic tests performed by the Division of Dairy and Livestock, Washington State Department of Agriculture. In every instance, our own studies showed the infection to be currently present and actively spreading through the herds. These studies included the demonstration of leptospiras, often in large numbers, in urine samples from the cattle and the isolation of *L. pomona* from dark field-positive urines.

Area 1.—Seven guinea pigs were employed in the examination of water samples collected from four sources on ranch 1 (table 1). Four were inoculated intraperitoneally with 2.0-ml. quantities of different samples. Three others were exposed by the cutaneous route. (Samples 1 and 2 were combined for exposure of 1 animal by the latter method.) The tabulated data (table 1) also include results obtained following blood passage from 4 of the guinea pigs in the first series to a second series of 4 animals.

Two hamsters, a young adult and a weanling, were also inoculated with each sample. The adults received 2.0 ml. of water intraperitoneally, the weanlings 1.0 ml. All but 1 became strongly seropositive within six weeks after inoculation. The negative animal was the weanling that received creek water.

Area 2.—This ranch, located about 60 miles from the former, was owned by the same rancher. The disease existed and was being studied simultaneously on both farms. The two ranches carried a herd of about 200 breeding cattle and the animals were often transported between the two. During late June and early July, 6 heifers and heifer calves in an experimental group of 30 animals in this area (area 2) were known to be excreting *L. pomona* in the urine. One continued to pass dark field-positive urine for several weeks. Groups of infected cattle had access to a small stream which passed through three pastures. The rate of flow in this stream (also in the stream in area 1) was estimated at 500 gal. per minute.

In September, samples of water were collected from this stream, one each from the upper, middle, and lower pastures. Weanling hamsters, young adult guinea pigs, and chicks, 10 to 14 days old, were inoculated immediately after collection of the samples. The animals were segregated by species and blood tests were made at intervals to detect serological developments.

Leptospira pomona was isolated from the blood of the guinea pig inoculated with water from the stream in the lower pasture on the eighth day when its rectal temperature had reached 105.0 F. Both the guinea pig and hamster inoculated with this sample developed high agglutination titers. The chicks, however, showed no evidence of infection.

TABLE 1—Recovery of Leptospira Pomona from Water Samples in Area 1 by Intraperitoneal Inoculation and by "Bathing" of Guinea Pigs

Source of sample	Method of inoculation	Results: original guinea pigs			Results: 1st-passage guinea pigs*			
		Temp.(F.) max.	DF exam.† peritoneal fluid	Titer (3rd wk.)	Temp.(F.) max.	DF. exam peritoneal fluid	Culture peritoneal fluid	Titer (3rd wk.)
Leaking hydrant pool (1)	Intraperitoneal	106.0(8d)§	.....	1:10	105.8(7d)	+(5d)	+	1:10
	Bathed‡	106.2(7d)	.....	1:10	105.8(5d)	+(3d)	+	1:10
Stock tank pool (2)	Intraperitoneal	105.8(7d)	.....	1:100	105.6(6d)	.....	....	1:100
Stock tank pool (3)	Intraperitoneal	106.0(7d)	+(10d)	1:10	105.4(7d)	+(5d)	+	1:10
	Bathed	106.0(7d)	.....	1:10	.....	.....	....	.....
Creek in pasture	Intraperitoneal	104.6(9d)	+(10d)	1:10	.....	.....	....	.....
	Bathed	103.8(9d)	.....	Neg.‖	.....	.....	....	.....

* The passage guinea pigs were inoculated intraperitoneally with blood specimens from the original guinea pigs when the latter were febrile. † Dark field examination. ‡ Samples from pools 1 and 2 were combined for exposure by "bathing." § (—d) = time in days intervening between date of inoculation and results as recorded. ‖ Animal probably killed too soon after inoculation to obtain a positive serological reaction; it showed evidence of relapse with secondary rise of temperature to 104.2 F. on the fifteenth day.

Low titer reactions of questionable significance appeared in hamsters and guinea pigs inoculated with the other water samples. Similar weak serological response occurred in 2 uninoculated guinea pigs housed in the same cage with the inoculated animals. This suggests that the doubtful reactions may have resulted from low-grade, cross infection contracted through exposure to the infected cage mates which had received lower pasture water.

Area 3.—On this ranch, located 100 miles or more from the other two, serological evidence of infection was fully confirmed by positive dark field findings in urine samples from 5 heifer calves and by subsequent recovery of *L. pomona* from these calves by inoculation of laboratory animals.

Samples of water collected from a large stream in the pasture and from a slough created by overflow from watering tanks in the corral were inoculated into hamsters, guinea pigs, and chicks. The hamsters and guinea pigs were isolated individually in order to prevent cross infection.

Both hamsters were serologically positive about five weeks after inoculation. The 1 inoculated with slough water was strongly positive and, on necropsy, *L. pomona* was isolated from kidney tissue. The hamster inoculated with river water showed a titer of 10^{-2}. It died from an injury and the tissues were not cultured.

The guinea pig and chicken inoculated with the river water died one and four weeks, respectively, after inoculation. Peritoneal fluid from the guinea pig gave a doubtful partial reaction at 10^{-1}. The tissues were not cultured. The guinea pig and chick inoculated with slough water remained serologically negative.

Area 4.—This area is separated from the others by distances of 150 miles or more. The presence of the disease in the herd was first suspected as the result of abortions. The diagnosis was made on the basis of positive serological tests and was confirmed by our studies. Leptospiras were demonstrated in appreciable numbers in the urine of 1 of 19 cows which had recently aborted. Ten of the 19 hamsters inoculated with urine samples from these cattle became seropositive for *L. pomona* within five weeks after inoculation. The urine of most of these cows contained agglutinins against *L. pomona* in relatively high titer. We have found that high titers correlate with severity of infection.

Of several water samples collected, only one seemed likely to yield leptospiras. This sample was obtained from a pool into which intermittent overflow from the one watering tank collected. The other samples were taken from pools and puddles of melted snow water more or less distant from the area where the cattle tended to congregate. Because of a temporary shortage of weanling hamsters, these samples were held for a week at room temperature before inoculation.

TABLE 2—Summary of Recoveries of Leptospira Pomona from Surface Waters

Ranch No.	Sources	Recoveries	Final criteria for identification
1	Stream	1	Serologically in animal.
	Pools(3)	3	Isolation, seroidentification.
2	Stream	1	Isolation, seroidentification.
3	Stream	1	Serologically in animal.
	Pool	1	Isolation, seroidentification.
4	Pool	1	Demonstration in kidney.*

* Levaditi-stained sections of kidney, from a hamster which died nine days after inoculation, showed morphologically typical spirochetes with characteristic distribution in the tissue.

One of the 2 hamsters inoculated with the tank overflow water died nine days after inoculation and Levaditi-stained sections of a kidney showed appreciable numbers of argentophilic structures, situated primarily in lumens of the tubules and interstitially. These organisms exhibited all the morphological characteristics of leptospiras.† The other hamster inoculated with this sample and those inoculated with the other water samples, were seronegative when tested about five weeks and again seven weeks after injection.

The results of the study are summarized (table 2).

DISCUSSION

Success in isolation of *L. pomona* from water appeared to have been correlated with conditions favoring heavy contamination with urine from infected cattle.

Five strains were isolated in pure culture and all were conclusively identified as *L. pomona* by the agglutination-lysis test using specific high titer antiserums. The identification of additional strains recov-

† We have never observed evidence of naturally acquired leptospirosis in the strain of hamster used (obtained from Lakeview Hamster Colony, Newfield, N. J.).

ered by animal inoculation, but not by culture, was established by demonstration in the serums of the infected hamsters or guinea pigs of agglutinins against a known strain of *L. pomona* or, in one instance, by demonstration of morphologically typical spirochetes in the kidney of the inoculated hamster.

That the infection was rapidly spreading through the herd on the first ranch (area 1) was indicated (1) by a series of official (state laboratory) serological tests made at four- to seven-week intervals throughout the summer which showed a 20 to 30 per cent increase in the number which were positive on each successive blood test and (2) by the prevalence of individual animals excreting relatively large numbers of leptospiras in the urine as confirmed by dark field examination of urines in the field. Two field trips were made 13 and 22 days after collection of the water samples. On the first trip, urine samples were collected from 17 "reactor" heifers, segregated on the basis of a herd test made about six weeks previously, and 10 were positive on dark field examination. On the second trip, urine samples were collected from 14 of the group of animals that were negative to the last blood test and, on dark field examination, six were positive and two were questionable. About 26 per cent of the latter group became serologically positive during this interval. Some of the cattle were eliminating the organisms in large numbers, estimated as up to millions per milliliter of urine. One animal, a heifer about 4 months old, in the latter group showed enormous numbers of leptospiras, perhaps 100 million per milliliter of urine, on three successive daily examinations. The numbers were greater than are ordinarily obtained in cultures under favorable conditions.

All water samples were thoroughly examined by dark field. Concentration by centrifugation was not attempted because of finely divided materials present. One morphologically typical Leptospira was found in the pool at the base of one stock tank (sample 2) and, two weeks later, leptospiras were seen in water from another similar pool in the barnyard. This pool may have served as the source of infection for chickens and other animals, since several horses and swine and 1 sheep on the ranch were shown to be serologically positive. One of the horses was known to have become positive during the period when the disease was epizootic among the cattle.

Blood samples from 12 chickens, about 3 months old, were tested for agglutinins against *L. pomona* shortly after the organism was observed in the overflow water which served as their water supply. Four of the 12 birds were positive, 1 with a titer of 10^{-3} and 3 with 10^{-5}. About one month later, blood tests on another group of older chickens, which had been confined away from the barnyard water a short time before, were negative except for a few equivocal reactions in the lower dilutions.

Subsequent experience in experimental infection of chickens suggests that the susceptibility of the chick is transient, being relatively high for only the first few days of life. The water may have become contaminated after most of the chicks had become sufficiently mature to resist infection.

Conditions in area 3 appear to have been less favorable for recovery of the organism from water. Although most of the group of 40 heifers examined were serologically positive, only 5 were excreting leptospiras. On this ranch, the size of the stream (the Walla Walla River near its confluence with the Snake) and the rate of flow of water ‡ at the point where the sample was collected made recovery of the organism the more surprising. However, this was the only source of water for a large number of cattle on this ranch and the possibility of pollution by infected herds upstream can not be excluded.

In area 4, conditions also appeared unfavorable. The cattle had been vaccinated with a *L. pomona* bacterin several weeks before, and had been treated with streptomycin two days before, we visited the ranch. Such treatment, especially the latter, might be expected to reduce the shedding of leptospiras. Furthermore, the water samples were held (on a window sill) for a week at temperatures fluctuating between 10 to 18 C. before inoculation into hamsters. In spite of this, a pathogenic Leptospira, presumably *L. pomona*, was detected in one sample.

Other factors, which probably contributed to success in recovering the organism,

‡ According to U. S. Geological Survey records,[5] obtained at a station located 2 or 3 miles upstream from the point sampled, the flow on the day of sampling averaged 252,100 gal. per minute.

were that the surface waters in this region (the Columbia Plateau) are regularly alkaline, favoring their survival and, with one exception, they were recovered during the dry season (July–October) when the dilution of urine in streams would be minimal.

A series of attempts to again recover the organism from the streams in areas 1 and 2, beginning three weeks after collection of the original (positive) samples and extending over periods of two and four months, respectively, was unsuccessful. This failure may have been partially due to (1) changes in herd management directed toward reduction of exposure to contaminated water, or (2) subsidence of the epizootic, or to both.

No cattle had been pastured along the stream passing through area 1 for about ten days prior to the first sampling, yet the organism was still present. Also the evidence suggests that the peak of the epizootic had passed before the stream in area 2 was first sampled. This may indicate that *L. pomona* does not persist, at least in numbers sufficient to be easily detected, for more than a few weeks in rapidly flowing streams which are not subject to continual, heavy contamination.

Unfortunately, no attempt was made to determine the length of time that the organisms will survive in naturally infected water when stored under different conditions. However, field observations suggest that the organism may persist in surface water for at least ten days. This was substantiated when creek water was artificially contaminated with urine from a shedder animal and it showed actively motile leptospiras on dark field examination 15 days later.

SUMMARY

Five strains of *Leptospira pomona* were recovered from surface waters associated with cattle with leptospirosis in four areas. The sources included: confluent pools surrounding a leaking irrigation hydrant, pools or sloughs (3 flowing and 1 stagnant) created by overflow from stock tanks, and natural swift-flowing streams (3).

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Keys to the Identification and Differentiation of the Immature Parasitic Stages of Gastrointestinal Nematodes of Cattle

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DIKMANS [11] reported that 25 nematode species occur in the alimentary tract of cattle in the United States.

However, recent reports and surveys by Porter,[19] Baker,[4] Koutz,[16] Cooperrider,[9] Cooperrider *et al.*,[10] Threlkeld and Bell,[28] Bailey and Herlich,[5] Bailey and Thorson,[6] Andrews *et al.*,[2] Vegors *et al.*,[27] and Dikmans and Kates[12] indicate that the nematodes most commonly found in the gastrointestinal tract of cattle in various representative regions in this country number only 11 species. Three of them are normally located in the abomasum: the twisted stomach worm, Haemonchus spp.;* the medium stomach worm, *Ostertagia ostertagi;* and the small hair worm, *Trichostrongylus axei.* Seven of the remaining eight are normally located in the small intestine: the small intestinal worms or cooperids, *Cooperia punctata, Cooperia pectinata,* and *Cooperia oncophora;* the small hair worm, *Trichostrongylus colubriformis;* the threadworm, *Strongyloides papillosus;* the thread-necked strongyle, *Nematodirus helvetianus;* and the hookworm, *Bunostomum phlebotomum.* Although the adults of the eleventh species, the nodular worm, *Oesophagostomum radiatum,* occur in the cecum, large intestine, and colon, immature stages of this species may be found in the small intestine, especially in the ileum.

Investigations reported by Andrews and Maldonado,[2] Basir,[3] Douvres,[13,14] Herlich,[15] Mönnig,[17] Sprent,[22] Stewart,[23] and Threlkeld [24] have provided descriptions of the life histories and of the immature parasitic stages of all of these species, except *Haemonchus placei, C. pectinata,* and *C. oncophora.* Although the life history of *S. papillosus* was determined in the rabbit,[7] the specific characteristics of the developing nematodes undoubtedly are not appreciably affected by the identity of the host.

Recent studies by Bailey and Herlich,[5] Vegors *et al.*,[27] and others reveal that heavy loads of immature stages along with adult worms are encountered in the bovine gastrointestinal tract at necropsy. Although the identification of these immature stages is of practical importance, it usually has not been carried out, partly because of a lack of sufficient information concerning their structure and partly because keys for their differentiation have not been available. Four keys, which are considered to be of value for this purpose, are presented in the present paper. They specifically provide for identification of the third-stage larvae of eight species and the fourth-stage larvae of nine of the 11 species, according to the most common location of the larvae in the host: one key each for the third- and fourth-stage larvae in the abomasum and one key for the third- and fourth-stage larvae in the small intestine. As third-stage hookworm larvae are located in the lungs, a description of this stage is omitted. These keys do not provide for the identification of the immature fifth-stage worms because specimens in this stage have characteristics similar to those of the mature adults and can be identified from the species descriptions given by Ransom,[20] and others. Due to the lack of a description of the life history of *H. placei,* the characteristics of the immature parasitic stages used in the keys for this species are based on those made for *H. contortus* in sheep.[26] Since Stewart's [23] description of immature *C. punctata* from cattle is essentially like Andrews'[1] description of *Cooperia curticei* from sheep, it is possible that the anatomical characteristics used in the present keys to distinguish *C. punctata* from the other parasites inhabiting the intestine may also well serve to identify *C. pectinata* and *C. oncophora,* at least as to genus.

Except in one instance, where a measurement is used, the keys are based entirely on diagnostic anatomical characters. Only those characters which can be easily discerned, primarily the anatomy of the anterior and posterior ends, are used. The use of measurements was avoided in constructing the keys, partly because the major body measurements of the immature

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* Recent evidence by Roberts *et al.*[21] and Bremner [8] has shown that Haemonchus from cattle differs morphologically and cytologically from the Haemonchus found in sheep, consequently constituting a distinct species. Roberts *et al.*[21] have retained *Haemonchus contortus* as the name for the species occurring in sheep and have proposed the name *Haemonchus placei* (Place, 1893) for the species occurring in cattle.

stages of the various species are in many cases closely similar, or overlapping, and partly to make the keys as time-saving and efficient as possible. The descriptive terms used in the keys are either taken from the published life history descriptions or were originated by the present writer. The keys and drawings (plate 1) are largely based on the appearance of the larvae in median optical section.

Only those characters used in the keys are illustrated in the figures given in plate 1. The writer [14] reported that the structure of the anterior ends and the location and appearance of the excretory pore of the third- and fourth-stage larvae of *T. axei* and *T. colubriformis* were identical; therefore, figures 1, 3, and 3A apply to both species. He [14] also observed that the tail ends of the third- and fourth-stage larvae of *T. axei* and of early fourth-stage larvae of *T. colubriformis* were identical; hence figure 2 applies to the aforementioned stages of both species. Basir [7] reported that there was no difference in the appearance of the anterior ends of the third- and fourth-stage larvae of *S. papillosus;* therefore, figure 21 applies to both stages of this species. Herlich,[15] who described the life cycle of *N. helvetianus,* did not illustrate the anterior end of the parasitic third-stage larva; therefore, the anterior end of the infective third-stage larva (fig. 22) is used in the key. The figures are either enlarged copies from the published descriptions or original drawings by the author. The scale accompanying each figure in plate 1 is either original, copied from the published illustration, or derived from the measurements given in the published descriptions.

Keys for the Identification of Nematode Larvae

KEY TO THIRD-STAGE NEMATODES IN THE ABOMASUM

1) Buccal cavity negligible in size or not discernible; esophagus anteriorly truncated; excretory pore may be located in a conspicuous depression; tail with bluntly rounded end and may be bent ventrad (fig. 1 and 2) ..... *T. axei*
Buccal cavity 5 to 7 μ long and easily discernible; in other features differs from above 2
2) Buccal cavity tubular with conical base and partially surrounded by esophagus; projecting anteriorly from latter are 2 laterally situated processes; tail dactylate (fig. 4 and 7) ..... *O. ostertagi*

Legends for Figures in Plate 1—All Figures Are Lateral Views

Fig. 1—Anterior region of a parasitic third-stage larva of Trichostrongylus axei (original).
Fig. 2—Posterior end of a parasitic third-stage larva of T. axei (original).
Fig. 3—Anterior region of a fourth-stage larva of T. axei (original).
Fig. 3A—Anterior end of a fourth-stage larva of T. axei (original).
Fig. 4—Anterior end of the parasitic third-stage larva of Ostertagia ostertagi (original).
Fig. 5—Anterior end of the fourth-stage larva of O. ostertagi (original).
Fig. 6—Anterior end of the parasitic third-stage larva of Haemonchus contortus (after Veglia, 1915).
Fig. 7—Posterior end of the parasitic third-stage larva of O. ostertagi (original).
Fig. 8—Posterior end of the parasitic third-stage larva of H. contortus (after Veglia, 1915).
Fig. 9—Posterior end of the fourth-stage larva of O. ostertagi (original).
Fig. 10—Posterior end of the fourth-stage larva of H. contortus (after Veglia, 1915).
Fig. 11—Anterior end of the fourth-stage larva of H. contortus (original).
Fig. 12—Posterior end of the parasitic third-stage larva of Trichostrongylus colubriformis (original).
Fig. 13—Posterior end of the larva of the fourth molt of T. colubriformis (original).
Fig. 14—Anterior end of the parasitic third-stage larva of Oesophagostomum radiatum (after Andrews and Maldonado, 1941).
Fig. 15—Anterior end of the fourth-stage larva of Oe. radiatum (after Andrews and Maldonado, 1941).
Fig. 16—Posterior end of the fourth-stage larva of Oe. radiatum (after Andrews and Maldonado, 1941).
Fig. 17—Posterior end of the third-stage larva of Cooperia punctata (after Stewart, 1954).
Fig. 18—Anterior end of the third-stage larva of C. punctata (after Stewart, 1954).
Fig. 19—Anterior end of the fourth-stage larva of C. punctata (original).
Fig. 20—Anterior end of the fourth-stage larva of Nematodirus helvetianus (after Herlich, 1954).
Fig. 21—Anterior end of the third-stage larva of Strongyloides papillosus (after Basir, 1950).
Fig. 22—Anterior end of the infective third-stage larva of N. helvetianus (after Herlich, 1954).
Fig. 23—Anterior end of the fourth-stage larva of Bunostomum phlebotomum (after Sprent, 1946).
Fig. 24—Posterior end of the third-stage larva of S. papillosus (after Basir, 1950).
Fig. 25—Posterior end of the larva of the fourth molt of S. papillosus (after Basir, 1950).
Fig. 26—Posterior end of the third-stage larva of N. helvetianus (after Herlich, 1954).
Fig. 27—Posterior end of the fourth-stage larva of N. helvetianus (after Herlich, 1954).
Fig. 28—Posterior end of the fourth-stage larva of B. phlebotomum (after Sprent, 1946).

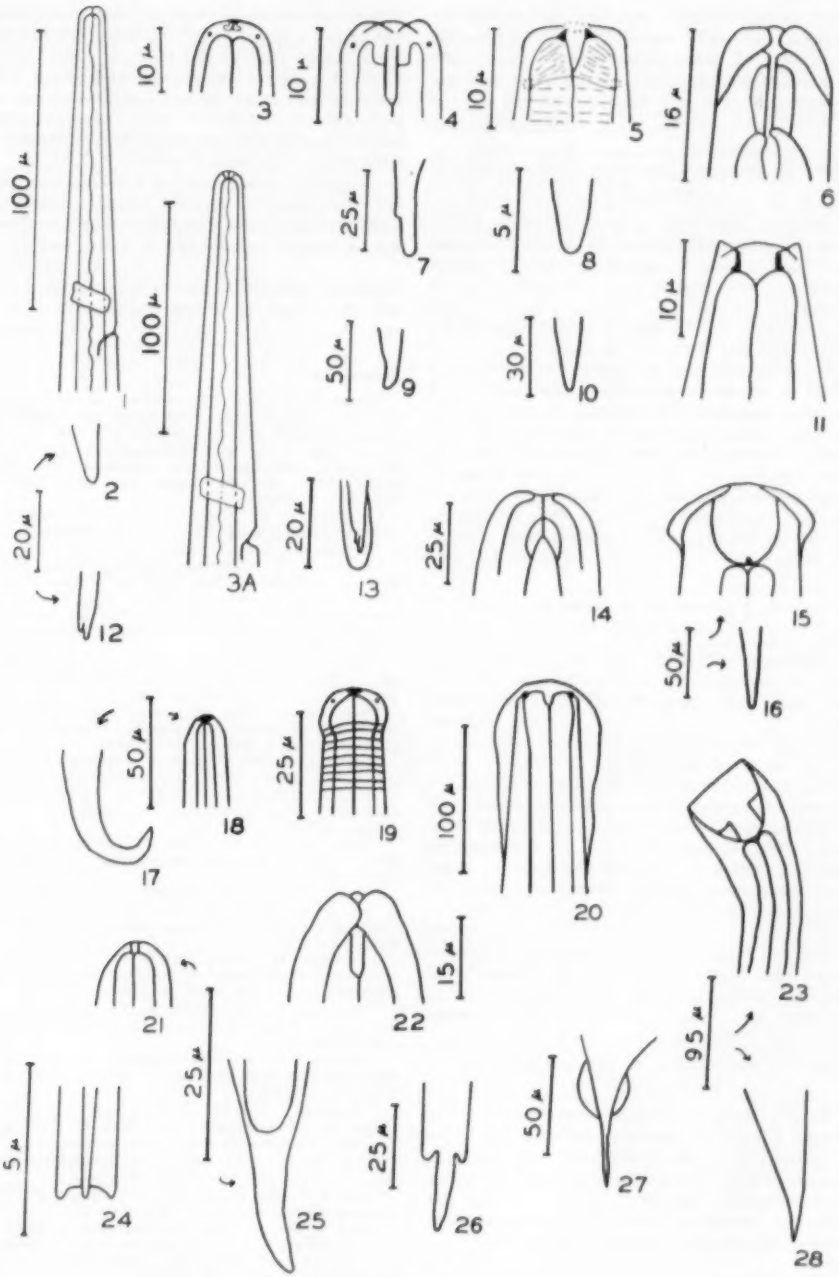
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Plate 1

Buccal cavity tubular and not surrounded by esophagus, its walls surrounded by 2 granular masses, each of latter shaped like a pincer; protuberance present on dorsal part of anterior end of esophagus; tail end bluntly rounded (fig. 6 and 8) ..... Haemonchus spp.

KEY TO FOURTH-STAGE NEMATODES IN THE ABOMASUM

1) Excretory pore located in conspicuous notch; buccal capsule wall appears as a bisected semilunar body or as 2 isomeric stubby J-shaped structures; tail end bluntly rounded (fig. 3, 3A, and 2) ..... *T. axei*
Excretory pore not located in conspicuous notch; in other features differs from above 2
2) Buccal capsule conical, appears anteriorly as 2 rodlike or toothlike structures, posteriorly as 2 thin lines completely surrounded by the esophagus; heavy diagonal striations present in anterior portion of esophagus; tail end smoothly rounded (fig. 5 and 9) ..... *O. ostertagi*
Buccal capsule globular in appearance, relatively deep and thick-walled; anterior end of esophagus not as above; tail attenuated and rounded (fig. 10 and 11) ..... Haemonchus spp.

KEY TO THIRD-STAGE NEMATODES IN THE SMALL INTESTINE

1) Esophagus anteriorly tapered; 2 conspicuous ovoid or pear-shaped vacuolated areas laterally flank buccal cavity and anterior end of esophagus (fig. 14) ..... *Oe. radiatum*
Esophagus anteriorly truncated; no such structures as above at anterior end 2
2) Tail end terminates in single process 3
Tail end terminates in more than one process 4
3) Tail attenuated and "characteristically bent dorsad" (fig. 17 and 18) ..... *C. punctata*
Tail has a dorsal and ventral lobe between which lies a rod-shaped process extending beyond the posterior margin of the lobes (fig. 22 and 26) ..... *N. helvetianus*
4) Tail end terminates in 2 tubercles of unequal lengths (fig. 1 and 12) ..... *T. colubriformis*
Tail end trifid, appearing as 3 prongs (fig. 21 and 24) ..... *S. papillosus*

KEY TO FOURTH-STAGE NEMATODES IN THE SMALL INTESTINE

1) With a cephalic cuticular inflation 2
Without a cephalic cuticular inflation 4
2) Esophagus anteriorly dilated and rounded; cuticular transverse striations in cephalic region very pronounced (fig. 19) ..... *C. punctata*
Esophagus anteriorly truncated; cuticular transverse striations in cephalic region not pronounced 3
3) Buccal capsule, small, appears tubular and unarmed; tail end spikelike, in the form of a finely attenuated terminal spine (fig. 20 and 27) ..... *N. helvetianus*
Buccal capsule, large, appears egg- or cup-shaped, and armed with a tooth at its base; tail end blunt, not spinelike (fig. 15 and 16) ..... *Oe. radiatum*
4) Buccal capsule cup-shaped, and armed with 4 cuticular lancets at its base; tail terminates in a point (fig. 23 and 28) ..... *B. phlebotomum*
Buccal capsule not cup-shaped or armed; tail terminates in other than a point 5
5) Excretory pore located in a conspicuous notch; tail end bluntly rounded (early fourth-stage larva) or terminates (in fourth molt) in 2 tubercles of unequal lengths (fig. 2, 3, 3A, and 13) ..... *T. colubriformis*
Excretory pore not located in a conspicuous notch; tail end trifid or "fingertip-shaped" (fig. 24 and 25) ..... *S. papillosus*

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Effect of Antibiotics on *Vibrio Fetus* and Bovine Spermatozoa

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VIBRIOSIS in cattle occurs in many parts of the world. Infected bulls serve as spreaders of the disease during coitus and by artificial insemination. A major problem which confronts operators of artificial breeding centers is to prevent the passage of *Vibrio fetus* to healthy cows via diluted semen. Use of antibiotic agents for the destruction of *V. fetus* is of primary concern, since this may lead to a greater number of pregnancies per service and, consequently, help control vibriosis. The work presented in this paper represents separate *in vitro* studies on the effect of several antibiotics, singly and in combination, against *V. fetus* and bovine spermatozoa.

Terpstra and Eisma [14] reported *V. fetus* as one of the causes of lowered fertility, *enzootische steriliteit*, in bulls and that infection may be transmitted from male to female at coitus or by the practice of artificial insemination. Frank and Bryner [6] reported consistent isolations of *V. fetus* from herds with a breeding history of venereal infection.

Antibiotics have been used extensively in diluted semen for the purpose of controlling bacterial growth and the spread of *V. fetus*. Adler and Rasbech [1] diluted semen with a sulfanilamide-egg yolk diluent containing streptomycin. Almquist *et al.*[2] found that 1,000 μg. of streptomycin per milliliter did not significantly affect the viability of bull spermatozoa. Bolton *et al.*[3] showed that dihydrostreptomycin in concentrations of 2,000 and 1,000 μg. per milliliter of diluted semen was satisfactory for artificial insemination. Foote and Bratton [5] reported that 2,000 μg. of polymyxins B, D, and E or 100 μg. of aureomycin ® could be added per milliliter of diluent without spermicidal effects. Stallcup and McCartney [13] found that 200 μg. of terramycin® per milliliter was not toxic to spermatozoa.

Plastridge and Easterbrooks [11] reported that 2,000 μg. of streptomycin per milliliter of diluent was required to destroy *V. fetus* when the diluent was stored at 5 C. Orthey and Gilman [10] stated that of three antibacterial agents, penicillin, sulfanilamide, and streptomycin, only the latter appeared to possess bactericidal activity against *V. fetus* in yolk-citrate diluent. McEntee [9] reported that the best and easiest way to control vibriosis is to breed artificially with noninfected semen or semen to which 500 units of penicillin, 500 μg. of streptomycin, and 3 mg. of sulfanilamide have been added per milliliter of diluted semen.

MATERIALS AND METHODS

Antibiotics used were dihydrostreptomycin sulfate (Merck), neomycin sulfate (myciſradin ® sulfate, Upjohn), polymyxin B sulfate (Pfizer), penicillin G sodium (Merck), streptomycin sulfate (Merck), terramycin hydrochloride (Pfizer), and aureomycin hydrochloride (Lederle). Dilutions of antibiotics were prepared with sterile glass distilled water in accordance with manufacturers' specifications. Antibiotic levels chosen were 2,000, 1,000, 500, 250, and 125 μg. (units for penicillin) per milliliter of diluent. Storage temperature for all tests was 5 C. Exposure intervals were placed at two, four, and eight hours and one, two, three, and four days.

Two separate experiments were conducted. One dealt solely with the effect of the antibiotics, singly and in combination, on bovine spermatozoa in skim milk; the other dealt with the effect of the antibiotics and their combinations on *V. fetus*-skim milk mixtures. Antibiotics were combined in accordance with a scheme proposed by Gunnison and Jawetz.[7]

Skim milk (autoclaved at 10-lb. pressure for 10 minutes) in 5-ml. amounts was used as the semen diluent. After sterilization, the pH of the milk was adjusted to 6.8 to 6.9 with Na_2HPO_4 (0.5 M) buffer. Semen was obtained from the Artificial Breeder's Cooperative, University of Maryland, College Park. The number of spermatozoa was determined by the hemacytometer method described by Coffin.[4] Average number of spermatozoa per cubic millimeter for 21 ejaculates was 1,030,000 with a range of variation from 670,000 to 1,500,000.

Semen was diluted 1:25 and 1:50 in antibiotic-diluent mixtures which were immersed in a water bath at 25 C. The water bath with tubes was then placed at 5 C. Cooling of tubes was gradual so as to avoid "temperature shock." Diluted semen without antibiotics served as controls and were

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handled identically with respect to conditions of the experiment.

After the desired exposure interval, diluted semen was examined for rate, type, and degree of motility under the microscope (x 250) at 37 C. A drop of the sample was placed on a preheated slide with coverslip and placed in the 37-C. incubator for 10 minutes. Ten microscopic fields were examined and the degree or intensity of motility was estimated and expressed in terms of percentage of motile sperm. Percentage readings were based on intervals of 10. For each antibiotic or combination of antibiotics, three separate trials (each trial prepared in duplicate) were run and the motility ratings from each trial were averaged.

The mean motility rating and the average estimated rate of motility for samples of freshly diluted semen were 67.0 and 3.1 per cent, respectively. The latter figure was obtained using an arbitrary scale where 4.0 was considered the maximum rate.

Three strains of *V. fetus* were used; two were isolated at the outset of the experiment and one was procured from Dr. P. A. Hansen of this laboratory. Stock cultures were maintained in fluid thioglycollate medium (Hansen *et al.*[8]) stored at room temperature.

The inoculum for antibiotic-diluent mixtures was grown in fluid thioglycollate medium. Following three to four days of incubation, cultures were centrifuged at 1,500 r.p.m. for 20 minutes. The cell crop was washed with sterile saline (0.85%) and recentrifuged. Two additional washings were made. After the final washing, the density of the suspensions was adjusted to No. 5 of the McFarland nephelometer. The amount of inoculum for tubes containing *V. fetus*-antibiotic-diluent mixtures was 0.2 ml. These tubes were handled in the same manner as tubes containing semen-antibiotic-diluent mixtures.

A simple washing procedure was used to remove the organisms from contact with the antibiotic. Approximately 0.1 ml. of *V. fetus*-antibiotic-diluent mixture was added to 5.0 ml. of sterile saline. The tube was centrifuged in an angle centrifuge (Servall) for 15 minutes at 3,500 r.p.m. The supernatant was withdrawn aseptically and discarded. Five milliliters of saline was added and the sediment was resuspended and recentrifuged. The supernatant was again withdrawn and discarded and to the sediment was added 2.0 ml. of fluid thioglycollate medium. Tubes were incubated at 37 C. for 14 days and observed at daily intervals for growth. Doubtful tubes were examined microscopically and later subcultured to fresh thioglycollate medium.

Control tubes containing no antibiotic were handled in the same manner as *V. fetus*-antibiotic-skim milk mixtures.

RESULTS AND DISCUSSION

Dihydrostreptomycin.—Different levels of dihydrostreptomycin were added to diluted semen to observe the effect of the antibiotic on spermatozoan motility. The addition of 2,000 μg. of dihydrostreptomycin per milliliter of diluted semen was spermicidal. Following four hours of storage, a marked increase in the number of dead spermatozoa was observed. Forward

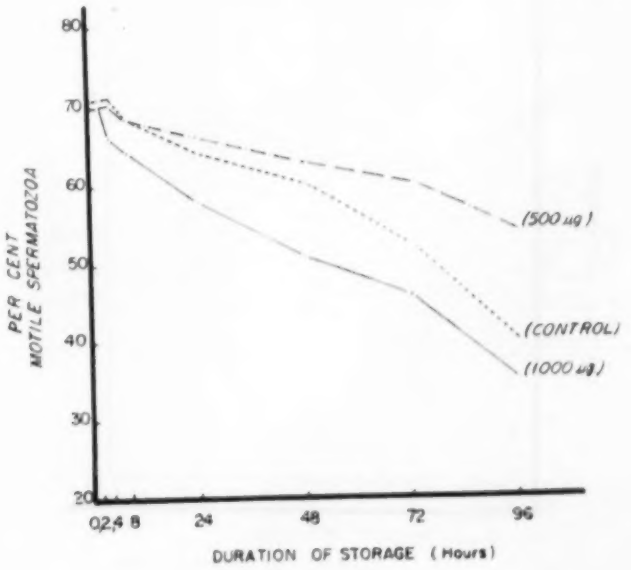

Fig. 1—Effect of 1,000 and 500 μg. of dihydrostreptomycin per milliliter on diluted bovine semen.

motion was retarded and motility was vibratory and oscillatory. At the 1,000-μg. level, dihydrostreptomycin was not as toxic as the 2,000-μg. level. After one-day storage, the sperm exhibited undulating movement with slow progression. An appreciable reduction in motility was noted following two days of storage. The motility was reduced approximately 25 per cent between one and four days (fig. 1). However, during the same period, at the 500-μg. level, motility was reduced only 10 per cent. The majority of spermatozoa showed vigorous forward motion following three days of storage. This level of dihydrostreptomycin appeared to be the most suitable level used.

The antibiotic was effective against *V. fetus*. Organisms could not be recovered from *V. fetus*-skim milk mixtures containing 500 μg. of dihydrostreptomycin per milliliter after two hours of exposure.

Streptomycin.—Addition of 2,000 μg. of streptomycin per milliliter of diluted semen reduced the motility of spermatozoa appreciably following eight hours and one day of storage. Forward motion was slowed considerably. At the 1,000-μg. level, however, motility was not markedly affected up to one day of storage but, following two days, a significant reduction in motility was noticed and spermatozoa exhibited undulating motion with slow forward progress.

Streptomycin at the 500-μg. level was found satisfactory in diluted semen up to two days of storage. Longer storage resulted in a 10 to 15 per cent drop in motility and a decrease in the rate of forward movement (fig. 2). Higher motility ratings were obtained when diluted semen was treated with 250 μg. of streptomycin per milliliter. At this level, for the entire four-day storage period, motility was better in all respects when compared to controls containing no antibiotic.

Vibrios could not be recovered from *V. fetus*-skim milk mixtures containing 500 μg. of streptomycin per milliliter after eight hours of exposure. At the 250-μg. level, however, *V. fetus* was recovered after four days of storage. Considering that *V. fetus* was not destroyed by streptomycin at the 250-μg. level, the most suitable level of streptomycin for use in diluted semen was 500 μg. per milliliter. However, semen treated in this manner could not be stored more than two days without spermicidal effects.

Neomycin.—Neomycin at different levels was added to diluted semen. In general, the antibiotic appeared to be relatively nontoxic to spermatozoa. A decrease in sperm motility was observed at the 2,000-

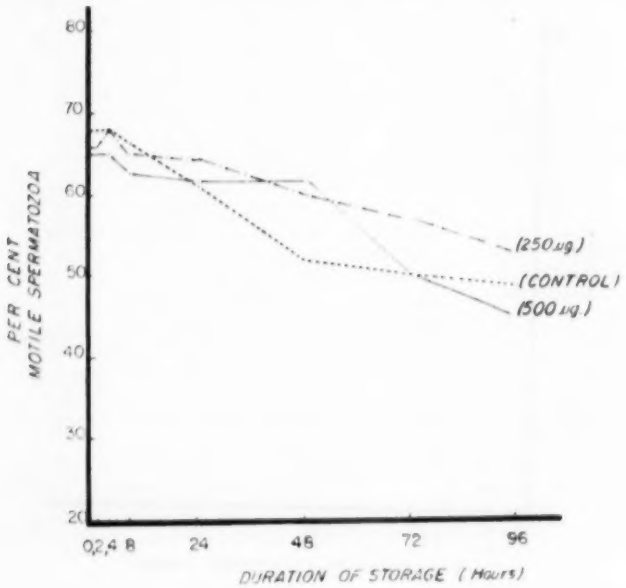

Fig. 2—Effect of 500 and 250 μg. of streptomycin per milliliter on diluted bovine semen.

μg. level following one to two days of storage. However, at the 1,000-μg. level, toxicity was not as marked. A slight reduction in the number of motile sperm did occur after two and three days of storage, with motility ratings being consistently lower than in controls.

Neomycin at the 500-μg. level was not spermicidal (fig. 3). Following three days of storage, motility was rapid and progressive and only a 15 per cent drop in motile sperm was observed. Close correlation between motility ratings of treated samples and controls is evident from the figure.

Addition of 500 μg. of neomycin per milliliter of skim milk was relatively effective against *V. fetus*. Organisms could not be recovered from the diluent following eight hours of storage, with the exception of one strain which was consistently recovered following one-day exposure. Neomycin at the 1,000-μg. level may be used in diluted semen; however, semen treated as such should not be stored more than two to three days.

This level proved to be very effective against *V. fetus* in skim milk, since vibrios could not be recovered after two hours of exposure to the antibiotic.

Penicillin.—Penicillin is widely used in semen diluents, usually in combination with other antibiotic agents. Most workers agree that penicillin in a concentration of 500 units per milliliter of diluent is satisfactory with respect to semen toxicity. According to results obtained in this experiment, penicillin at the 500-unit level in skim milk-diluted semen was not toxic to spermatozoa, and, apparently, at this level, the reduction of the percentage of motile sperm was much less than that which occurred in controls or in samples treated with 1,000 units of the antibiotic (fig. 4). At the higher level, following two and three days of storage, motility ratings were lower than in controls. During early periods of storage, no immediate toxic effects were observed at the two levels mentioned in the figure.

Penicillin in a concentration of 500 units per milliliter was relatively effective against *V. fetus*. Vibrios could not be recovered from the diluent after eight hours of exposure with the exception of one strain which was inactivated only after one day of storage. These results were unexpected, since penicillin is known to require active cell division to exert its bactericidal action. However, at refrigerator temperature, active cell division does not occur. The possible explanation offered is that the antibiotic probably exerted its greatest action against *V. fetus* during the cooling process of the samples.

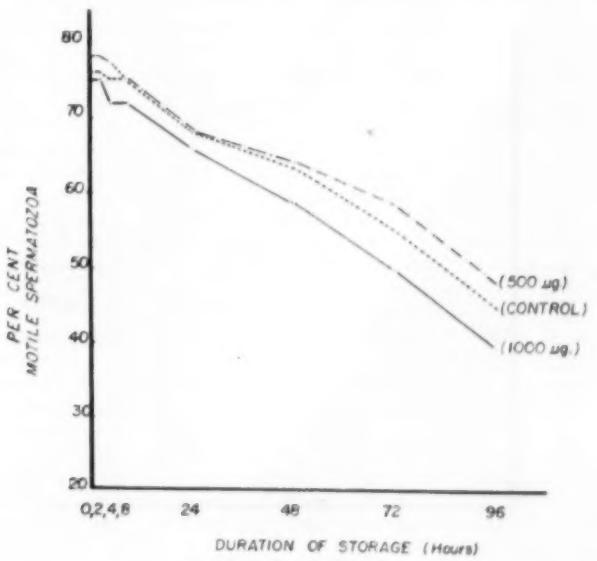

Fig. 3—Effect of 1,000 and 500 μg. of neomycin per milliliter on diluted bovine semen.

Polymyxin B.—Polymyxin was found to be the least spermicidal antibiotic agent used in this study. At high levels, the antibiotic was not toxic to spermatozoa. A moderate reduction in motility was observed at the 2,000-μg. level following two days of storage; however, additional storage resulted in a marked decrease in the percentage of motility.

Polymyxin at the 1,000-μg. level appears to be the maximum concentration of the antibiotic which may be added to diluted semen without spermicidal effects (fig. 5). After three days of storage, the spermatozoa appeared rapid and progressive and the number of motile sperm approximated 50 per cent. Motility ratings of controls and of samples treated with 1,000 μg. of polymyxin per milliliter were closely correlated.

Polymyxin at the 2,000- and 1,000-μg. levels was not effective against *V. fetus*. The organisms apparently resisted the action of the antibiotic in skim milk for up to four days of storage.

Aureomycin and Terramycin.—These antibiotics were used singly and were found toxic to spermatozoa at low levels (250 and 125 μg./ml.), although aureomycin (chlortetracycline) was less toxic than terramycin (oxytetracycline).

Both antibiotics were ineffective against *V. fetus* in skim milk. Vibrios were recovered from the diluent containing 250 and 125 μg. each of aureomycin and terramycin following four days of storage. Conditions of this experiment were not conducive to the stability of aureomycin, since this antibiotic is known to be most active and stable in acid pH ranges.

According to the results obtained, aureomycin and terramycin are unsatisfactory as antibacterial agents for use in skim milk-diluted semen. However, Stallcup and McCartney [13] and Foote and Bratton [5] found that 200 μg. of terramycin or 100 μg. of aureomycin per milliliter, respectively, could be used in egg yolk-diluted semen without spermicidal effects. The disagreement in results may be due to the type of diluent used. It must be borne in mind that the proper choice of antibiotic and the most suitable level for use in diluted semen will rest primarily on the type of semen diluent used.

In most instances, an apparent improvement in motility ratings was obtained during later periods of storage in samples treated with optimum levels of the relatively nontoxic antibiotics as compared to controls containing no antibiotics. This was probably due to the action of the antibiotic on bacteria which were capable of interfering with sperm motility.

Antibiotic Combinations.—Penicillin in a concentration of 500 units per milli-

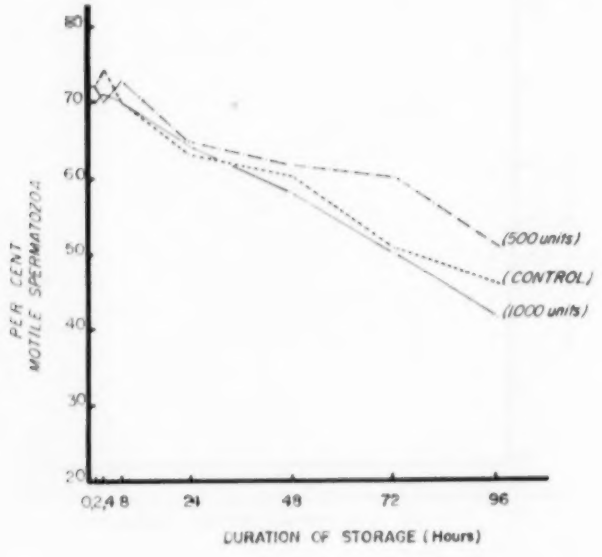

Fig. 4—Effect of 1,000 and 500 units of penicillin per milliliter on diluted bovine semen.

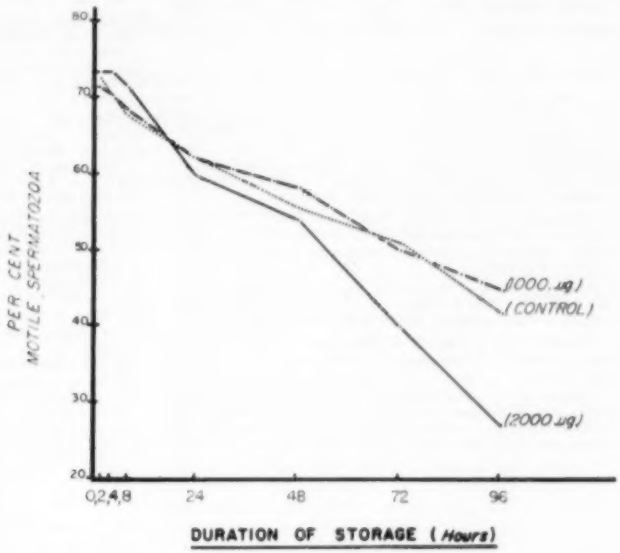

Fig. 5—Effect of 2,000 and 1,000 µg. of polymyxin per milliliter on diluted bovine semen.

liter was combined with optimum levels of streptomycin, dihydrostreptomycin, and neomycin (250 µg., 500 µg., and 500 µg., respectively). None of these combinations were toxic to spermatozoa in the concentrations employed. The combinations did appear to exert a more pronounced effect against *V. fetus* than any of these antibiotics acting alone at similar levels. Organisms could not be recovered from the diluent following two hours of exposure as a result of treatment with these combinations.

Since polymyxin showed little spermicidal activity and has been reported by Foote and Bratton [5] to be highly bacteriostatic or bactericidal, or both, this antibiotic was used in combination with several antibiotic agents. The authors found no reports in the literature where polymyxin has ever been used in combination with other antibiotic agents for treating semen in artificial insemination. Polymyxin at the 1,000-µg. level was combined with 500 µg. per milliliter each of streptomycin, dihydrostreptomycin, and neomycin. None of these combinations exhibited spermicidal effects in diluted semen. All were more effective against *V. fetus* than either of the antibiotics alone, with the exception of the polymyxin-neomycin combination. This combination appeared to be no more effective against *V. fetus* than neomycin alone.

Summary

Various antibiotics at different levels, singly and in combination, were added to semen diluted at a rate of 1:25 and 1:50 with skim milk to determine optimum levels for spermatozoan motility.

Optimum levels for spermatozoan motility were 500 µg. of dihydrostreptomycin, 250 µg. of streptomycin (500 µg. was satisfactory up to 2 days), 500 µg. of neomycin, 500 units of penicillin G, and 1,000 µg. of polymyxin B. Aureomycin ® and terramycin ® were highly toxic to spermatozoa at low levels.

Optimal concentrations (as regards sperm viability) of each antibiotic were added to *Vibrio fetus*-skim milk mixtures to determine their effect on *V. fetus*. Dihydrostreptomycin and streptomycin (500 µg./ml.) were more effective against *V. fetus* than were neomycin, penicillin, polymyxin, aureomycin, and terramycin. The most suitable single antibiotic used in this study with respect to sperm toxicity and destruction of *V. fetus* was dihydrostreptomycin (500 µg./ml.).

The antibiotic combinations used were not spermicidal. The combinations exerted a greater effect against *V. fetus* than either of the antibiotics acting alone, with one exception; polymyxin and neomycin com-

bined were no more effective than neomycin acting alone.

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The Efficiency of the Hotis Test in the Identification of Streptococcic Infections of the Udder

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THE HOTIS TEST has been used generally as a diagnostic method for detecting streptococcic mastitis in dairy cattle. Its use in conjunction with other simple tests has been effective in tentatively identifying *Streptococcus agalactiae*. Widespread use of penicillin and other antibiotics in the treatment of mastitis in recent years has led to a decreased incidence of cases due to *Str. agalactiae*.[2, 6] This decrease in the significance of *Str. agalactiae* has been accompanied by some increase in the number of cases due to other mastitis streptococci. This study was made to identify the microorganism found in infected udders in contemporary herds and to determine the contribution of the Hotis test in testing programs in those herds.

REVIEW OF LITERATURE

The use of the original Hotis test described by Hotis and Miller[5] was clarified by Murphy,[11] Miller,[10] and Schalm.[15] Schalm incubated samples at 37 C. for 15 to 20 hours without additional incubation, because longer incubation permitted a large percentage of samples infected with *Streptococcus dysgalactiae* and *Streptococcus uberis* to become positive to the Hotis test. To simplify recording and interpreting the results, Schalm developed a key for the Hotis and the microscopic tests and a classification key for diagnosis of mastitis. Using this simple procedure, *Str. agalactiae* infections were reduced to a low level or were completely eradicated from several herds through regular herd tests, segregation of infected animals, and treatment of involved quarters with therapeutic agents.[16] Most investigators have agreed that *Str. agalactiae* was the dominant organism of bovine mastitis. Plastridge and Hale[14] reported that 37.5 per cent of 14,205 cows were infected with *Str. agalactiae* on initial herd tests. This represented about 70 per cent of all mastitis detected in herds in Connecticut. The bulk of the mastitis research for the past 25 years has been concerned primarily with the control of this microorganism. Plastridge[12] and Schalm[17] concluded that it could be eradicated completely from individual herds by a program of systematic testing and treating.

In a five-year study which included 2,684 clinical cases of mastitis, Hughes[8] found 25 per cent of the infections due to *Str. agalactiae*, 18 per cent to *Str. uberis*, 13 per cent to Micrococcus, 12 per cent to *Str. dysgalactiae*, 8 per cent to Corynebacterium, 13 per cent to other microorganisms, and 11 per cent were negative or contaminated samples. These results clearly indicated the significance of all of the mastitis streptococci. Burkey and Bouma[2] reported that *Str. dysgalactiae* and *Str. uberis* were not found in infected quarters in a herd which had not been given antibiotic treatment. In this herd, 76 per cent of the infections were due to *Str. agalactiae*. In a similar herd where infected quarters had been treated with penicillin for four years, only 19 per cent of the infections were due to *Str. agalactiae*, while 52 per cent were due to *Str. dysgalactiae*, *Str. uberis*, and other streptococci. These reports and numerous others have emphasized the role of all streptococci in clinical mastitis. Schalm,[17] in a recent review, did not consider streptococci, other than *Str. agalactiae*, significant in herd health problems, even in herds free of *Str. agalactiae*. In these California herds, *Micrococcus pyogenes* had become the major mastitis organism.

MATERIALS AND METHODS

The bulk of samples used in this study were collected at monthly intervals from the herd at the University of Tennessee and at various intervals from other herds. Just before each cow was milked, the udder was washed with 200 to 400 p.p.m. of available chlorine water and dried with a dry cloth. Two to three streams of milk were discarded into a strip cup. Samples were collected from each quarter after the tip of the teat had been scrubbed with a pledget of cotton soaked in 70 per cent alcohol. Approximately 9.5 ml. of milk was collected, using aseptic precautions, into a sterile vial containing 0.5 ml. of 0.5 per cent bromocresol purple solution. Strip-cup results and alkaline reactions to bromocresol purple were recorded. The same procedure was followed on cows with acute attacks of mastitis which occurred during the month in the herd of the University of Tennessee. These and other cows shown, by monthly tests, to be definitely infected were treated at 24-hour intervals with four intramammary infusions of penicillin.[7]

Properly collected samples were transported to the laboratory and placed in a 37 C. incubator

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in wire racks. After incubation for 18 to 21 hours, the results of the Hotis test were interpreted according to Schalm,[15] with some modification. With the exception of the tests of one herd infected with *Str. agalactiae*, typically positive Hotis tests were seldom seen. The majority of the reactions was suspicious or atypically positive. Suspicious tests consisted of a yellow cream line with a greenish yellow zone just below the cream line or a yellow or greenish yellow sediment in the bottom of the tube which did not adhere when the tube was inverted, or both. Occasionally, the entire column of milk would turn an olive drab color. Atypically positive reactions usually were similar to the suspicious reactions with a yellow sediment but with a large accumulation of sediment which adhered firmly when the tube was slowly inverted two or three times.[11] Usually the sediment had a flocculent appearance and was faintly greenish yellow, or a granular appearance and was canary yellow. Typical yellow flakes or balls of growth on the sides or bottom of the tubes were rarely seen.

Milk films of all samples were prepared from the incubated milk.[8] Microscopic examinations were made for the identification of the predominating types of microorganisms present. The average number of leukocytes found in not less than ten fields was recorded and the approximate number of leukocytes per milliliter was calculated. Samples containing streptococci or one million leukocytes per milliliter of milk in the absence of a predominating microorganism were streaked over one fourth of the surface of an Edwards' blood agar plate.[16] After incubation at 37 C. for 15 to 18 hours, plates were examined and the types of colonies and changes in the medium were recorded. Usually, the plates were re-examined after 42 hours of incubation. Colonies thought to be streptococci were picked and inoculated into tryptose phosphate broth, with 10 per cent sterile cow's blood serum added. After proper incubation, the serum-broth cultures were used to inoculate differential mediums. Occasionally, it was necessary to repeat the plating and picking operations to assure pure cultures.

Essential differentiation mediums were selected for identification procedures. One drop of an 18-hour serum-broth culture was added by pipette to the following mediums: two sets of litmus milk for incubation at 10 C. and 45 C., tryptose broth containing 6.5 per cent sodium chloride, 1:5,000 methylene blue milk, arginine broth, sodium hippurate broth, and aesculin broth. The preparation of these mediums has been described by Little and Plastridge.[8] To confirm the results obtained from these biochemical tests, fermentation tests were made using the following sugars: sucrose, lactose, sorbitol, mannitol, raffinose, trehalose, inulin, and salicin. The basal medium for the sugars contained 1.0 per cent tryptose, 0.5 per cent sodium chloride, 0.1 per cent disodium phosphate, and 1 ml. of 1.6 per cent alcoholic bromocresol purple solution per liter.[8] Ten milliliters of a 10 per cent sterile sugar solution was added to 100 ml. of basal medium. Five per cent sugar solutions were used in the case of trehalose, salicin, and raffinose.

All tests were incubated at 37 C. for at least 48 hours except as indicated above. Growth at 10 C. was checked at the end of one and two weeks. Some difficulty was experienced in obtaining growth in sodium hippurate broth and a few of the sugars. This was partially overcome by using only freshly prepared mediums and incubating for 60 hours. Boiling the medium in a water bath for 10 minutes to evacuate dissolved oxygen was resorted to on occasions when freshly prepared medium was not available.

The cows were classified as to the presence of mastitis on the basis of direct and indirect tests. The indirect tests, palpation, strip cup, alkaline reaction to bromocresol purple, and leukocyte count, were used to indicate inflammation. The direct tests, Hotis test, microscopic examination of incubated samples, and reaction on Edwards' medium, were used to indicate infection. If one or more of the indirect tests were positive and the direct tests clearly indicated infection, the cow was considered to have an established infection. In addition, cows with persistent infections of three or more months' duration, as indicated by the direct tests, were considered to have established infections.

RESULTS

Four herds were tested in preliminary work, and cultures from all samples containing streptococci were isolated and identified. There were no quarters infected with *Str. agalactiae* in herds B, C, and D, and also *Str. dysgalactiae* was not found in herds C and D. The results of the Hotis test on samples containing streptococci in the preliminary work are summarized (ta-

TABLE 1—Hotis Test Reactions According to Types of Streptococci in One Herd Infected with Streptococcus Agalactiae and in Three Herds Free of Streptococcus Agalactiae Infection

Organisms	Hotis test	Samples in Herd A (No.)	Herd B (No.)	Herd C (No.)	Herd D (No.)
Str. agalactiae	Typically pos.	14			
	Atypically pos.				
	Suspicious	2			
	Negative	1			
Str. dysgalactiae	Typically pos.				
	Atypically pos.	3	2		
	Suspicious	1	1		
	Negative				
Str. uberis	Typically pos.			1	
	Atypically pos.		5	7	4
	Suspicious		4	6	5
	Negative	3	2	3	1
Str. viridans or enterococcus	Typically pos.	1	2	1	
	Atypically pos.			3	3
	Suspicious		2	3	3
	Negative				3

TABLE 2—Hotis Reactions Produced by Streptococci for 12 Months in a Herd Free of Streptococcus Agalactiae

	Typically positive Hotis		Atypically positive Hotis		Suspicious Hotis		Negative Hotis		Totals	
	Established infections (No.)	Transitory infections (No.)	Established infections (No.)	Transitory infections (No.)	Established infections (No.)	Transitory infections (No.)	Established infections (No.)	Transitory infections (No.)	(No.)	(%)
Str. dysgalactiae	26	0	38	8	35	7	6	17	137	42
Str. uberis	13	0	33	9	24	12	13	9	113	35
Str. viridans or enterococcus	11	0	15	14	10	11	5	8	74	23
Totals	50	0	86	31	69	30	24	34	324	100

ble 1). Of 25 cows in herd A, 18 (72%) were infected in 25 quarters. Milk samples from 14 (82%) of the 17 infected with *Str. agalactiae* and the one infected with *Streptococcus viridans* developed typically positive Hotis reactions (table 1). Two infected with *Str. agalactiae* developed suspicious reactions and one a negative reaction. Of 52 cows in herd B, 16 (31%) were infected in 18 quarters. Milk samples from only two of the 18 quarters showed typically positive Hotis tests, and the causative organisms were identified as an enterococcus and *Str. viridans*. The other 16 gave atypical Hotis tests. Of 33 cows in herd C, 12 (36%) were infected in 24 quarters. As in herd B, milk samples from only two quarters showed typically positive Hotis tests. Those from other quarters gave atypical results. Of 47 cows in herd D, 13 (28%) were infected in 19 quarters. None of the samples gave typically positive Hotis tests. Thus, the Hotis test detected 14 of 17 (82%) of the *Str. agalactiae* infections, 5 of 7 (71%) of the *Str. dysgalactiae* infections, 17 of 41 (42%) of the *Str. uberis* infections, and 10 of 21 (48%) of the *Str. viridans* and enterococcus infections.

A more detailed study of the Hotis test reactions found in herd B (University of Tennessee) was made over a 12-month period (table 2). Of 2,887 samples tested, 324 contained streptococci. Of the 324 samples, 137 (42%) were *Str. dysgalactiae*, 113 (35%) were *Str. uberis*, and 74 (23%) were *Str. viridans* or enterococci. For all samples from which streptococci were isolated, the Hotis test was positive in 50 tests (15%), atypically positive in 117 tests (36%), suspicious in 99 tests (31%), and negative in 58 tests (18%). In established infections, *Str. dysgalactiae* had more of a tendency to produce positive Hotis tests than the other streptococci. In addition to the 167 positive Hotis tests reported in streptococcic infections (table 2), 56 tests (25% of all positives) were produced by Micrococcus. *Streptococcus dysgalactiae* had a tendency to produce a Hotis reaction with a flocculent, faintly greenish yellow sediment, and *Str. uberis* had a tendency to produce reactions with a granular appearance similar to that produced by some Micrococcus. No clear-cut lines could be drawn between these reactions as to species.

During the period covered by this detailed study, 37 cows had acute clinical cases of mastitis. The types of bacteria involved and the percentages of each were as follows: *Str. dysgalactiae*, 24 per cent; *Str. uberis*, 22 per cent; other streptococci, 5 per cent; Micrococcus, 19 per cent; Corynebacterium, 8 per cent; coliform, 5 per cent; Pseudomonas, 8 per cent; and no bacteria, 8 per cent. Thus, 51 per cent of the cases of clinical mastitis were due to streptococci. Of the established infections, as detected by routine tests, 28 per cent developed clinical mastitis in the 12-month period.

Discussion

The Hotis test was found to be a valuable diagnostic method for mastitis in a herd infected with *Str. agalactiae*, with a record of 82 per cent efficiency. These results compared favorably with those of other investigators.[2, 10, 11, 15] When the Hotis test was applied to herds relatively or entirely free of *Str. agalactiae*, a high percentage of the infected samples developed atypically positive or suspicious reactions. *Streptococcus dysgalactiae* had a tendency to produce reactions with a coarsely flocculent yellow sediment without flakes on the sides of the tubes. This probably was due to differences in agglutinin content, as suggested by McColloch and Fuller.[9] *Streptococcus uberis* had a tendency to

produce reactions with a finely granular yellow sediment indistinguishable from the reaction produced by some micrococci. This probably was due to the fact that *Str. uberis* frequently grew in short chains. There seemed to be some relationship between the length of the chain and the type of Hotis reaction. Indistinct reactions by these two organisms can be explained further by their lack of ability to ferment lactose readily. Streptococci must have the ability to produce sufficient acid from lactose to lower the pH to about 5.4 at 37 C. before characteristic Hotis test changes can occur.[10] Because different isolates of *Str. dysgalactiae* and *Str. uberis* usually lacked one or more of the characteristics just discussed, these species did not consistently produce a distinguishing reaction with the Hotis test.

Streptococci of the *viridans* and enterococcus groups at times produced all of the above reactions. Infections with these microorganisms are presumed to originate from fecal contamination. Since the teats were constantly exposed to fecal material, in spite of aseptic precautions, these organisms may at times appear in normal and abnormal milk samples and may be present in small numbers in the teat canal. While they have not been found to be an important cause of mastitis, in a few instances they have been associated with mild subclinical cases.[17]

During the period of this study, the relative proportions of *Str. dysgalactiae* and *Str. uberis* infections were the same in herd survey tests (table 2) and all clinical cases. A greater number of infections were reported by the herd survey method because many subclinical cases were included. Only 50 per cent of the established infections were detected by the Hotis test, but microscopic tests detected most of the remaining 50 per cent. *Streptococcus dysgalactiae* and *Str. uberis* caused more clinical cases of mastitis than all other organisms combined. Hughes,[6] in a survey of clinical cases in one veterinary practice over a five-year period, reported similar results and pointed out that herd survey studies tend to overemphasize the importance of organisms easily detected by such methods. The present work confirms his observation.

SUMMARY

The Hotis test used in conjunction with other methods was studied to determine its suitability as a diagnostic test for mastitis. The Hotis test was found to be an excellent method of detecting *Streptococcus agalactiae* infection in a herd. It was found to be of some value in detecting infection with *Streptococcus dysgalactiae* and *Streptococcus uberis*. However, due to the occurrence of a large number of negative and suspicious reactions and to the nature of the infection, it was not considered to be a good method for the detection of mastitis caused by the latter organisms. *Streptococcus dysgalactiae* and *Str. uberis* were found to be the most frequent causes of udder infections in three herds which were free of *Str. agalactiae*. In these herds, clinical mastitis occurred in about the same proportion as detected infections due to the various organisms identified.

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The Effect of Environmental Temperature on Blood Volume and the Antipyrine Space in Dairy Cattle

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LITERATURE is voluminous concerning the effect of environmental temperature on blood volume and composition [1-9] with particular reference to man, a profusely sweating species. According to the recent review by Bass and Henschel,[10] high ambient temperature increases blood and interstitial fluid volume; low temperature exerts an opposite effect.

The present report on blood volume, and also on body water, measured by antipyrine dilution concerns cattle. Cattle differ from man in being "slightly sweating" above 80 F. This is demonstrated by the fact that, at 105 F. ambient temperature, about 100 per cent of the heat produced by cows is dissipated by vaporization, while in man about 160 per cent of the heat produced is dissipated by vaporization (100 % of the heat produced plus about 60 % of the environmental heat absorbed by the body at 105 F.).[11]

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METHODS

Data were obtained in a climatic laboratory under four diurnally variable temperature rhythms: 10 to 40 F., 40 to 70 F., 60 to 110 F., and 70 to 100 F. The temperatures were controlled so that the troughs (low temperature) occurred at 5 to 7 a.m. and the peaks (high temperature) at 3 to 4 p.m. All of these data were obtained during the period of rising temperature. The blood volume determinations were completed approximately three hours before the temperature peak, while the antipyrine determinations were completed about the time of the temperature peak.

Nine cows were studied: 3 dry Jerseys, 3 lac-

TABLE 1—Blood and Serum Volume (Mean Values with Standard Deviation*)

Temperature condition	No. of cows	No. of determinations	Serum volume (cc./kg. body wt.)		Blood volume (cc./kg. body wt.)		Hematocrit (r.b.c. %)	
			Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Dry Jerseys								
Midwest cold (10 to 40 F.)	3	12	34.03	2.8	63.2	2.25	45.88	3.65
Midwest normal (40 to 70 F.)	3	12	36.64	3.67	62.01	5.05	40.88	3.41
Imperial Valley (60 to 110 F.)	3	9	40.16	3.95	68.19	11.87	40.72	3.59
Midwest hot (70 to 100 F.)	3	9	39.0	4.5	61.19	5.05	36.32	4.34
Lactating Jerseys								
Midwest cold (10 to 40 F.)	2	7	44.23	0.6	66.41	3.8	33.2	2.49
Midwest normal (40 to 70 F.)	2	8	47.06	4.67	68.84	7.0	31.56	2.65
Imperial Valley (60 to 110 F.)	3	5	49.2	7.44	72.82	9.09	32.56	3.33
Midwest hot (70 to 100 F.)	3	7	44.49	8.6	70.19	10.91	36.9	2.62
Lactating Holstein-Friesians								
Midwest cold (10 to 40 F.)	3	9	49.17	2.32	75.69	3.34	35.18	1.66
Midwest normal (40 to 70 F.)	3	12	54.49	5.65	84.38	9.76	35.3	2.0
Imperial Valley (60 to 110 F.)	3	5	58.4	6.64	91.56	7.28	36.32	3.54
Midwest hot (70 to 100 F.)	2	6	55.5	3.55	85.22	4.38	34.65	1.38

* Standard deviation $= \sqrt{\frac{\Sigma x^2}{n-1}}$ $\quad \Sigma x^2 = \Sigma X^2 - (\Sigma X)^2/n$

TABLE 2—Results of Analysis of Variance (These Values Were Computed on the Basis of a Two Criteria and Disproportionate Frequency Analysis)

Analysis of variance criteria	Source of variation	"F" values and significance		
		Serum volume	Blood volume	Hematocrit (% cells)
Breed and climatic condition	Among breeds	11.22 Sig. at 1%	13.94 Sig. at 1%	6.4337 Sig. at 5%
	Among climatic conditions	9.867 Sig. at 1%	15.84 Sig. at 1%	2.4492 Not sig.
	Interaction	1.3115 Not sig.	1.1529 Not sig.	41.98 Sig at 1%
Lactation status and climatic condition	Among lactation status	6.221 Sig. at 5%	6.077 Sig. at 5%	3.1304 Not sig.
	Among climatic conditions	10.67 Sig. at 1%	3.431 Sig. at 5%	8.0439 Sig. at 1%
	Interaction	2.318 Not sig.	2.118 Not sig.	3.5225 Not sig.

Note.—Analysis of variance method taken from Snedecor, G. W.: Calculation and Interpretation of Analysis of Variance and Covariance. Collegiate Press, Inc., Ames, Iowa (1934): 96 pp.

tating Jerseys, and 3 lactating Holstein-Friesians.

Serum volume was measured with the blue dye T-1824, a method widely used on man [12, 13] and a variety of animals,[14-17] including the cow.[18, 19] The animals were deprived of feed for 12 hours before the serum volume determinations and were not allowed water while the determination was in progress. During the injection of the dye and the withdrawal of the blood samples (both through a polyethylene catheter in the jugular vein), the animals were restrained with a rope halter; at all other times they were standing free in the stan-

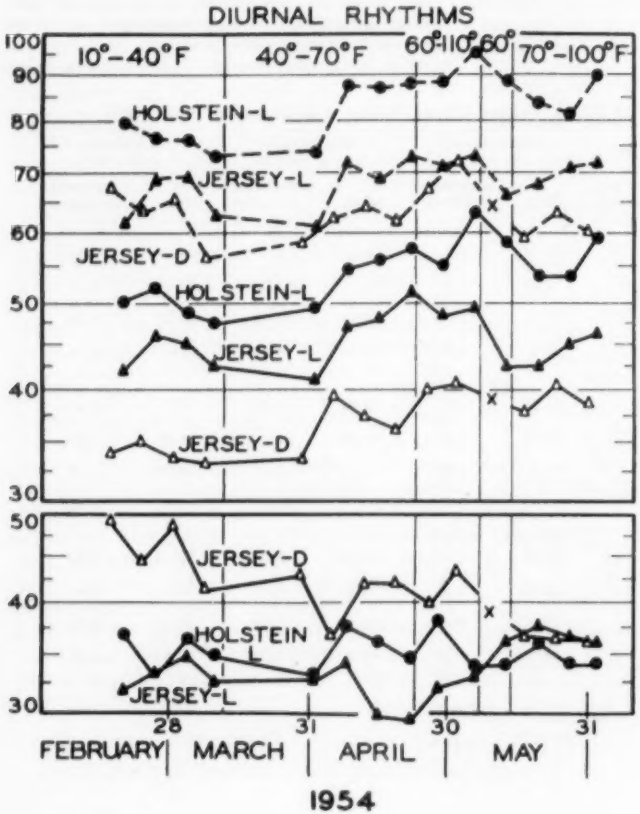

Fig. 1—Blood volume (broken line, upper 3 curves), serum volume (solid lines) in cubic centimeters per kilogram of body weight, and hematocrit (red blood cell percentage, bottom segment) plotted against time (involving 4 diurnal temperature rhythms, upper axis) on arithlog paper (on which equal percentages are represented by equal slopes).

Note that lactating (L) cows have higher blood and serum volumes but lower hematocrits than dry (D) cows; that Holstein-Friesians have higher blood and serum volumes than Jerseys; and that the blood and serum volumes tend to increase with increasing average temperature of the diurnal rhythm. Each line represents average values.

chion. The dye solution, T–1824, was injected at the rate of 20 mg./100 kg. of body weight for dry cows and 25 mg./100 kg. for lactating cows. Blood samples were withdrawn at approximately ten-minute intervals for one hour, and the samples were allowed to clot. The serum was obtained by centrifugation after which the densities of the samples containing the dye were read at 420 μ on a Coleman junior spectrophotometer. The hematocrit of heparinized blood was read after 30 minutes of centrifugation at 1,500 r.p.m. (radius 19 cm.); the leukocytes were included with the plasma fraction. No correction was made for trapped plasma (estimated at 6 % by Reynolds[18]) or for the difference between the venous and the general body hematocrit.[20, 21]

Antipyrine was used for the determination of total body water[22]; it was administered at the rate of 2.5 Gm./100 kg. of body weight. The injection and sampling procedures were in general similar to the blood volume technique; however, for the antipyrine determinations, blood was collected at approximately one-hour intervals for six hours after the injection. The concentration of antipyrine in the blood serum was determined by the method of Brodie *et al.*,[23] using a Beckman DU spectrophotometer. The percentage of serum water was determined by drying to a constant weight.

Results and Discussion

The over-all results for serum volume determination and blood volume calculation are summarized (fig. 1; tables 1 and 2). Three effects are apparent.

Effect of Temperature.—Increase of environmental temperature was associated with expanded serum and blood volumes in all three categories of cattle, *i.e.*, dry Jerseys, lactating Jerseys, and lactating Holstein-Friesians. As suggested by Bazett *et al.*,[2] this increased blood volume is probably related to the vascularity of the skin. The closing of arteriovenous anastomoses with resultant greater capillary circulation increases the total capacity of the vascular system, blood pressure falls, and fluid is moved from the tissue spaces to the blood. Yet, the pulse rate of cows usually declines until the environmental temperature surpasses 100 F., and a slow pulse is generally associated with a higher than normal blood pressure; presumably other factors are involved.

Effect of Lactation.—The mammary gland is more vascular than other tissues

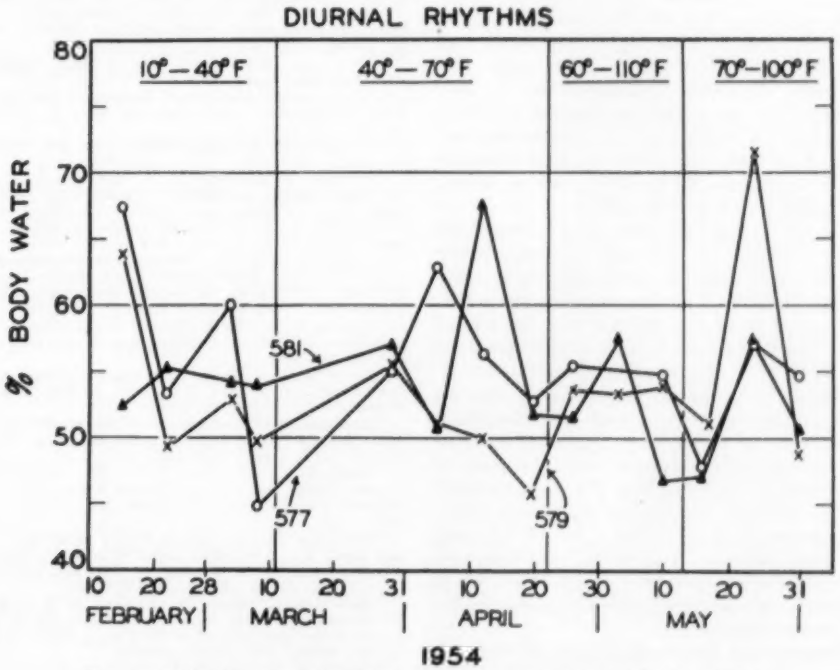

Fig. 2—Antipyrine space (total body water) as percentage of body weight.

of lower metabolic activity; yet, it seems improbable that this alone could account for the serum volume difference between dry and lactating Jerseys, a difference of 5 liters in a 1,000-lb. cow. It is more probable that the increased feed consumption and increased metabolism associated with lactation increase the vascularity of all of the visceral organs.

Effect of Breed.—Lactating Holstein-Friesians had larger serum and blood volumes in proportion to body weight than did lactating Jerseys. This breed difference is particularly striking during the 60 to 110 F. temperature rhythm, where it amounted to almost 20 cc. of blood per kilogram of body weight, over 2 gal. in a 1,000-lb. cow.

Total body water, determined by the dilution volume of antipyrine, ranged between 45 and 75 per cent of body weight; repeated at weekly intervals, the results on any one animal were so inconsistent as to be worthless (fig. 2).

SUMMARY

Serum and blood volumes were measured with the T-1824 hematocrit method in 3 dry and 3 lactating Jersey cows and in 3 lactating Holstein-Friesian cows. Total body water was estimated in the dry Jersey cows with the antipyrine dilution technique. All of the cows were exposed to each of four diurnally variable temperature rhythms: 10 to 40 F., 40 to 70 F., 60 to 110 F., and 70 to 100 F.

All the cows had increased serum and blood volumes at the higher temperature ranges; this was most noticeable in the lactating Holstein-Friesian cows with a difference of 9 ml. of plasma and 16 ml. of blood per kilogram of body weight between the 10 to 40 F. rhythm and the 60 to 110 F. rhythm. In lactating Jersey cows, the serum volume was 5 to 10 ml. and the blood volume 3 to 9 ml. per kilogram greater than were the serum and blood volumes of dry Jersey cows under the same conditions. In lactating Holstein-Friesian cows, the serum volumes were 5 to 11 ml. and the blood volumes 9 to 19 ml. per kilogram greater than were the serum and blood volumes of lactating Jersey cows under the same conditions.

Consecutive determinations of the volume of distribution of antipyrine were so variable as to lead to the conclusion that antipyrine does not consistently measure total body water in cattle.

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Studies on Arthritis in Swine
III. The Influence of Desoxycorticosterone in Normal and Arthritic Swine Due to Erysipelothrix Infection

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ARTHRITIS in swine is prevalent under farm conditions. In some cases, an infectious agent can be isolated, while in others no infectious agent can be found. Pyogenic bacterial infections produce enlarged, soft joints distended with pus.[5] This purulent type of infection occurs most frequently at the time of birth, probably through the umbilicus. In *Erysipelothrix rhusiopathiae*-infected joints, pus is usually absent but extensive proliferative changes may be present. In advanced chronic polyarthritis due to *Ery. rhusiopathiae*, the organism may disappear from the joints while the arthritis persists.[11] In such cases, many macroscopic and microscopic pathological changes similar to rheumatoid arthritis in man are present. Pannus formation over the articular cartilages with or without fibrous ankylosis, narrowing of the joint spaces with rarefaction of the adjacent bones, and greatly thickened joint capsules are evident.

Marked pathological changes in the adrenals and kidneys are often present.[11] These observations suggest that normal functions of the adrenal glands and kidney may be altered. These metabolic disturbances may possibly augment the arthritic condition. This belief prompted this series of experiments.

MATERIALS AND METHODS

The 24 experimental swine used in these experiments were from a purebred Chester White herd, established in 1941, in which erysipelas was not known to have occurred. They were 3 to 4 months old, weighed 75 to 125 lb., were in excellent health, and had been vaccinated against hog cholera, with anti-hog cholera serum and virus, at weaning time (8 weeks).

They were housed in pens with cement floors and were on pasture during the warm season. They were fed a well-balanced commercial ration which contained adequate minerals, vitamins, fat, and protein.

To study the effect of hormonal therapy on normal and arthritic swine, cortisone* and desoxycorticosterone acetate (DCA) were given subcutaneously. One gram of DCA was suspended in 20 cc. of sesame oil, while the cortisone was a saline suspension of cortone® acetate.

Soft tissues to be studied for histopathological changes were taken at the time of necropsy and fixed in 10 per cent formalin. Paraffin sections were cut and stained by the usual hematoxylin and eosin procedure. Bone specimens were decalcified according to Lillie's method,[8] sectioned, and stained with hematoxylin and eosin.

RESULTS

1) Repeated Intravenous Exposure of Swine Which Resisted One Exposure to Erysipelothrix Rhusiopathiae.—Four 90-lb. shoats, 4 months old, which were injected intravenously with 10 cc. of a 24-hour culture of the organism, resisted the exposure. Beginning ten days later, each was given 10 cc. of culture in the anterior vena cava, weekly for nine weeks. One died after the sixth injection and showed vegetative endocarditis involving the greater portion of the right ventricle. All of the leg joints were inflamed and the joint capsules were distended with cloudy fluid. The kidneys had many healed infarcts. The other viscera appeared normal.

A second animal died two days after the last injection and showed extensive vegetative endocarditis and infarcts in the kidneys. Bacteriological cultures made from the hearts and carpal and tarsal joints of both animals yielded *Ery. rhusiopathiae*.

The remaining 2 shoats developed chronic polyarthritis and were slaughtered five and seven months following the final intrave-

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* The cortisone used in these experiments was supplied by Merck and Co., Inc., as a grant.

nous injection. The first one killed showed proliferation of synovial villi, pannus formation in the carpal and tarsal joints, and extensive vegetative endocarditis involving both the tricuspid and bicuspid valves. The kidneys were mottled with numerous fibrotic areas, and the adrenal glands were cystic.

The one killed at seven months showed marked enlargement and rigidity of the carpal and tarsal joints and synovitis, proliferation of villi, and pannus formation in all leg joints. There were a few eroded areas in the articular cartilages. The synovial fluid was scanty. There were pronounced exostoses of the articular processes of a number of vertebrae. The adrenals were enlarged and had numerous clear cystlike structures in the cortex. The kidneys were gray and fibrotic, whereas all other viscera appeared normal. Bacteriological cultures from the joints yielded *Ery. rhusiopathiae*.

It is of interest that 3 of the 4 shoats receiving repeated intravenous injections of *Ery. rhusiopathiae* developed extensive vegetative endocarditis. Also, 2 of the 4 with arthritis had cystlike formations in the adrenal glands and gray infarcts uniformly present in the kidneys.

2) The Use of Hormonal Therapy to Alter the Course of Arthritis in Swine.—The adrenal hormones have a number of profound physiological effects in the body. Cortisone has been used extensively in the treatment of rheumatoid arthritis in man. Aside from its effects on carbohydrate metabolism and depletion of lymphatic tissue, it reduces inflammation of the joints and lessens the stiffness and discomfort. According to Turner,[12] DCA is concerned principally with the salt and water balance of the body. It aids in potassium excretion and sodium retention. The tissue culture experiments of Mancini and de Lustrig [7] have shown that it also has a stimulating effect on fibroblasts, whereas cortisone acetate had no effect.

The object of this series of experiments was to observe the effects of DCA alone, and of DCA administered simultaneously with cortisone, on normal swine and on swine with acute arthritis experimentally induced by *Ery. rhusiopathiae*.

A) Use of DCA and Cortisone in Normal Swine.—Group 1 consisted of 2 shoats which received, subcutaneously, 1 Gm. of DCA twice weekly until death occurred.

Group 2 consisted of 2 shoats which received, subcutaneously, 500 mg. of DCA twice weekly for 25 weeks.

Group 3 consisted of 2 animals which received, subcutaneously, 1 Gm. of DCA twice weekly and daily doses of 75 mg. of cortisone for 12 weeks.

One shoat in group 1 developed polydipsia, polyuria, and a ravenous appetite during the second week of treatment. It became paralyzed and died on the twenty-first day of therapy. At necropsy, the kidneys were swollen and congested with numerous ecchymoses in the cortex. The adrenals were enlarged and hemorrhagic. An excess of clear cerebrospinal fluid was present, and there was an increase of synovial fluid in the tarsal and carpal joints. All other tissues appeared normal.

The second shoat was slaughtered after seven weeks because of a paralytic condition. During the third week of treatment, it developed a pronounced polydipsia, polyuria, and a ravenous appetite which continued until incoordination and paralysis developed. During the fifth week, it became lame in all legs and would stand for only a few minutes at a time. The lameness became more marked during the sixth week and paralysis and the loss of coordination and of detectable reflexes developed on the last days before slaughter. During the last week, the water intake and the polyuria were reduced. Serosanguineous effusions distended the joints and some proliferation of the synovial villi was observed. The kidneys were infarcted and the adrenals were markedly atrophic. The cerebrospinal fluid was thin, clear, and excessive, and the meninges were hyperemic and edematous.

The second group of animals which received the lower level of DCA (500 mg.) developed polydipsia, polyuria, and ravenous appetites during the fourth week. These persisted until the time of slaughter. A slight soreness of the feet or legs may have been due to excessive weight. Both animals appeared healthy and gained in weight. At necropsy, the kidneys were mottled and showed infarcts and the adrenals were atrophic. All other viscera and the joints appeared normal.

The animals of the third group (2 Gm. of DCA weekly and 75 mg. of cortisone

daily) developed polydipsia, polyuria, and ravenous appetites during the third week, which persisted until the twelfth week when they were slaughtered. During the sixth week, both developed acute arthritis and refused to stand except for a few minutes while eating and drinking. The tarsal joints were enlarged and sensitive. The swelling subsided but the lameness persisted. Both animals otherwise appeared healthy and grew normally.

At necropsy, the kidneys of both shoats showed infarcts and the adrenals were atrophic. All other viscera appeared normal. The carpal and tarsal joints appeared normal in size, but mild synovitis, proliferation of the synovial villi, and beginning pannus formations were present. There was some erosion of the articular surfaces of the tarsal bones.

The bacteriological findings in joints and visceral organs of all animals receiving hormones were negative.

It appeared that the administration of DCA and DCA combined with cortisone resulted in adrenal atrophy. Treatment with these adrenal hormones as well as repeated intravenous exposure to *Ery. rhusiopathiae* produced mottled kidneys with infarcts. The administration of 75 mg. of cortisone daily prevented paralysis, loss of reflexes, and cerebrospinal fluid changes in the swine receiving the high level of DCA (2 Gm. weekly). However, this level of cortisone failed to prevent early arthritic changes in the joints which presumably were produced by DCA overdosage.

B) Use of DCA in Acute Arthritis Produced by Erysipelothrix Rhusiopathiae.—Each of 9 shoats received 10 cc. of a 24-hour culture of *Ery. rhusiopathiae* intravenously. As soon as they had developed acute polyarthritis (10 days to 3 weeks) with joint effusions, the DCA treatments were started.

The first group of 7 animals each received 1 Gm. of DCA in 20 cc. of sesame oil subcutaneously, twice weekly; the last two were given 500 mg. twice weekly.

All 7 animals in group 1 developed polydipsia, polyuria, and a ravenous appetite during the fourth week of treatment. Six of the 7 began to lose weight in the fifth or sixth weeks; the carpal and tarsal joints enlarged and the lameness increased. Only 1 was able to rise without assistance by the ninth week, and it recovered spontaneously.

One animal with chronic polyarthritis was killed after 98 days of treatment. The carpal and tarsal joints were enlarged and rigid and showed synovial villi proliferation and pannus formation. Bacteriological cultures from these joints were positive

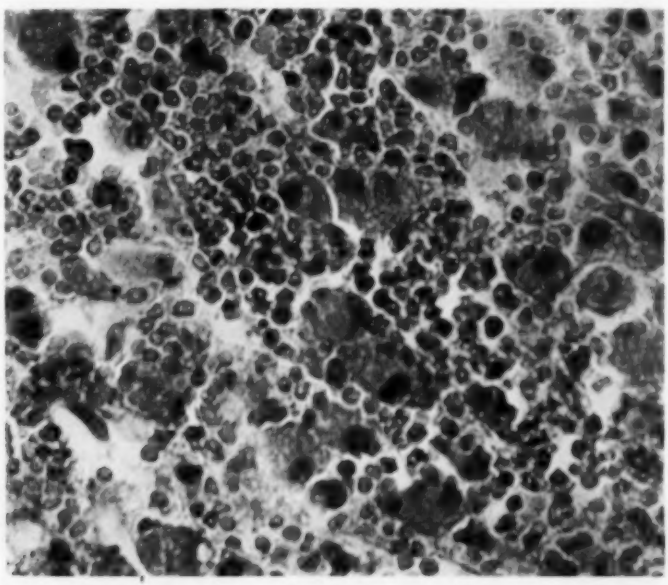

Fig. 1—Extensive hemorrhage in the cortex of the adrenal gland in acute swine erysipelas. x 680.

for *Ery. rhusiopathiae*. There were infarcts in the mottled kidneys and the adrenals were atrophic. All other viscera appeared normal.

The remaining 5 animals developed advanced polyarthritis and were killed four to five months after the beginning of DCA therapy. *Erysipelothrix rhusiopathiae* was isolated from the joints of 4 of the 5. One shoat showed advanced ankylosing spondylitis in addition to the pathological changes described in the preceding animal. Atrophic adrenal glands were present in all 6 which had received 1 Gm. of DCA twice weekly.

In the 2 shoats, which received 500 mg. of DCA twice weekly, a similar clinical and pathological course developed. At necropsy, after 12 and 17 weeks of hormonal therapy, the adrenals of both animals were atrophic and *Ery. rhusiopathiae* was isolated from their carpal and tarsal joints.

At the dosages used, DCA seemed to augment the development of arthritis rather than to have a therapeutic effect.

C) Use of DCA Administered Simultaneously with Cortisone for the Treatment of Acute Polyarthritis Due to Erysipelothrix Rhusiopathiae.—Intravenous exposure with 10 cc. of a 24-hour culture of *Ery. rhusiopathiae* resulted in acute arthritis in 5 shoats. Four were treated simultaneously with DCA and cortisone beginning two weeks postexposure; 1 was untreated.

The first group of 2 animals was given, subcutaneously, 1 Gm. of DCA twice weekly and 75 mg. of cortisone daily. The 2 shoats in the second group were given 500 mg. of DCA twice weekly and 50 mg. of cortisone daily.

Both animals in the first group showed definite clinical improvement after the second week of treatment and a further decrease of the lameness and swellings of the carpal and tarsal joints after four weeks. One appeared to have recovered after six weeks of therapy and the other was only slightly lame after eight weeks.

The 2 animals which received the lower level of steroid therapy showed clinical improvement after three weeks. A remis-

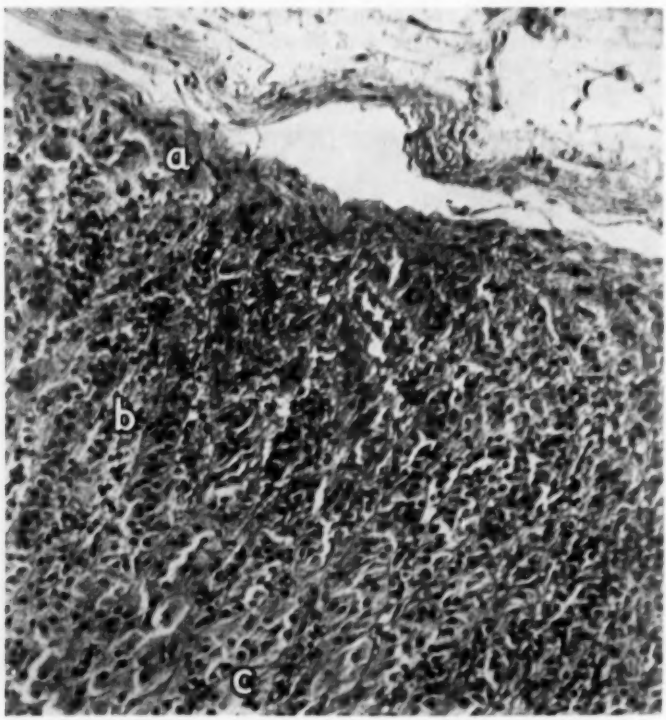

Fig. 2—The zona glomerulosa (a), the zona fasciculata (b), and a portion of the zona reticularis (c) of the adrenal gland of a hog with acute swine erysipelas, showing numerous foci of round cells. x 200.

sion of the acute symptoms, and some reduction of the joint swellings had occurred by the sixth week. Slight lameness persisted in 1 animal, apparently due to a unilateral carpitis.

The untreated animal developed chronic polyarthritis, became moribund, and at necropsy, eight weeks postexposure, showed a marked synovitis with an increased amount of turbid synovial fluid. Marked proliferation of the synovial villi and thickened joint capsules were found in most of the joints of the legs. The adrenals were enlarged and the kidneys were mottled and contained infarcts. All other viscera appeared normal. *Erysipelothrix rhusiopathiae* was isolated from the carpal and tarsal joints.

OBSERVATIONS ON PATHOLOGICAL CHANGES

The pathological changes varied. In swine which died of acute septicemia, pathological changes observed at necropsy were not different from those in other acute septicemic diseases. The lesions most pathognomonic of swine erysipelas were in the skin.

In the kidneys, subcapsular hemorrhages were sometimes present, depending upon the acuteness of the disease. There was evidence of acute inflammation, of degenerative changes in the parenchyma, and of interstitial hemorrhage. Infarcts were frequently found, some necrotic and others cicatrized.

In the swine which developed vegetative endocarditis lesions (part 1), bacteria and fibrin made up the vegetative masses in the early stages. Later, connective tissue proliferation and vascularization developed so that the vegetative masses became firm. Emboli originating from these masses on the endocardium probably resulted in infarcts in some distant organs, particularly the kidneys. Arteritis with plaque formation occurred in some cases.

The adrenals were enlarged and subcapsular hemorrhages were frequently observed (fig. 1) in acute swine erysipelas.

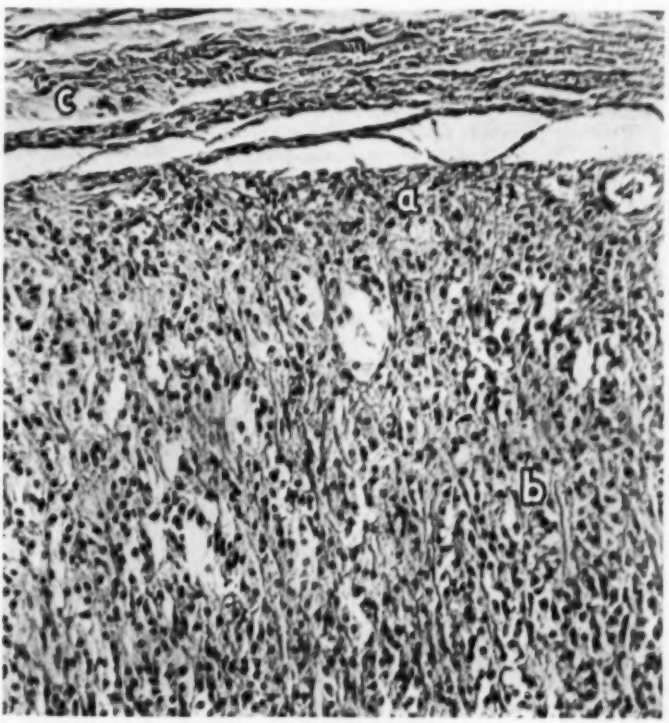

Fig. 3—The zona glomerulosa and a portion of the zona fasciculata of the adrenal gland from a hog which had experimentally produced chronic arthritis. The zona glomerulosa (a) is nearly obliterated. The fascicular zone (b) shows atrophy of the parenchymatous cells and numerous spaces. The capsule (c) appears edematous. x 200.

As the disease continued (2 months or more), the adrenals were frequently twice the normal size, soft, and swollen. The zona glomerulosa was infiltrated with leukocytes, and this zone sometimes partially disappeared. Numerous foci of round cells were scattered throughout the zona fasciculata and reticularis (fig. 2). It is not certain that these foci always result from *Ery. rhusiopathiae* infection. The adrenal medulla in all cases appeared normal. In advanced cases of chronic arthritis, a disruption of the normal architecture of the zona glomerulosa and reduction in thickness frequently occurred (fig. 3). In cases where the clear cystlike areas were present in the cortex, a true membrane did not surround these areas (fig. 4). The normal production of adrenal cortical hormones probably was interfered with in these cases.

DISCUSSION

The probable course of events in arthritis due to infection is as follows: infection is established in the joints causing an inflammatory reaction; edema and congestion of the synovial membranes occur, followed by proliferative changes in the intra-articular structures; the suppuration which does rarely occur is focal; and numerous lymphocytes and plasma cells, with a limited number of polymorphonuclear leukocytes and a few eosinophils, invade the area. Eventually, the synovial membrane assumes the appearance of granulation tissue, and individual villi resemble granulomatous polyps. The synovial fringes probably combine with proliferating perichondrium to form a pannus. The articular cartilage becomes eroded apparently by the pannus attachments and by the inflammatory reaction in the subchondral bone. Bacteria may disappear from the joints but the arthritic process may continue unabated.[11]

Adrenal involvement seemed to occur during the bacteremia and simultaneously with the invasion of the joints by the bacteria. The first changes observed in this gland were extensive hemorrhage (fig. 1) followed by leukocytic and round cell infiltration in the cortex (fig. 2). Marked enlargement of the gland frequently occurs with some disruption of the normal architecture. Cystlike formations (fig. 4) occurred in some cases but it is not known what relationship these had to arthritis. The zona glomerulosa was largely obliterated in some instances (fig. 3). Atrophy of the adrenal is a frequent finding in swine with advanced chronic arthritis which are suffering severe inanition. Normal adrenal function is probably altered by: the effects of illness, pain associated with body movements, degenerative changes of the kidney, subsequent impaired renal function, and the constant demand for glucocorticoid. It would seem that kidney and adrenal malfunction may influence the course of the disease and possibly contribute to the continued active manifestation of arthritis.

A comparison of the arthritis of swine and rheumatoid arthritis in man shows some similarities. According to Collins and Goldie,[2] the anatomic changes in the joints show clearly the following macroscopic and microscopic similarities: the pathological changes are essentially proliferative, nonsuppurative, and have a

Fig. 4—Cross sections of cystlike areas in cortex of the adrenal gland removed from a hog with experimentally produced chronic arthritis. x 5.

tendency toward granulomatous proliferations in the synovial membrane; pannus formation and infiltration of the tissue with lymphocytes occur forming focal collections; destruction of cartilage at the site of pannus attachment and subchondral cellular reactions appear; and intra-articular fibrous adhesions and rarefaction of the bone as revealed by radiography are common.

Collins and Goldie [2] believe that no further analogy can be drawn between the two diseases until the cause of rheumatoid arthritis in man is known. They also believe that one of three things may happen in advanced chronic arthritis of swine in which no infectious agent can be isolated and the arthritic condition continues unabated. (1) The lesion may resolve and the joint recover. (2) Healing of the damaged tissues may take place by fibrosis and this may include formation of intra-articular adhesions. (3) The synovial granulation tissue may assume certain properties of a benign neoplasm and proliferate in the absence of the exciting microorganism. Thus, the arthritis is maintained in a state of clinical activity.

The results of these experiments support this latter belief, especially in the case of the continued active clinical form when the joints are apparently sterile. It may be that kidney and adrenal pathological changes play a part in these cases of advanced chronic polyarthritis.

Fibrotic lesions in the kidneys and changes in the cortex of adrenal glands were the two most constant changes observed in the viscera of animals with advanced cases of chronic polyarthritis. The significance of these changes in the development of the arthritic syndrome has not been determined. It is known that reduced renal activity delays the excretion of a number of body wastes. Just what effect these waste products have on arthritis and on the adrenal gland is subject to speculation.

It was believed that if a disproportionate secretion of the adrenal cortex occurred in the arthritic animals with demonstrable adrenal changes, a similar pathological condition might be created in the joints of normal pigs by interfering with the normal hormonal balance of the adrenal gland through overdosage with exogenous hormone.

Adrenal atrophy was produced in the albino rat by Carnes *et al.*,[1] using repeated doses of DCA. Ragan *et al.*[5] observed polydipsia and polyuria in dogs receiving 25 mg. of DCA each day. They suggest that this could have been due to increased extracellular sodium concentration. No description of kidney damage was given. (It would seem that prolonged treatment could produce definite lesions in the kidneys.)

In this investigation, the 2 normal shoats which were given 2 Gm. of DCA per week died on days 21 and 49. This indicated that a profound physiological disturbance had occurred. The occurrence of polydipsia and polyuria, with adrenal atrophy in these animals supports the above observations.[1, 5] The 2 normal shoats which received 1 Gm. of DCA weekly developed polydipsia and polyuria but survived without other observable effects. On a body weight basis, this dosage is considerably less than the 10 mg. per day dose reported by Hartman and Brownell [4] as producing adrenal atrophy in the rat.

The normal hogs which received 2 Gm. of DCA per week plus 75 mg. of cortisone daily showed clinical signs of arthritis by the sixth week of therapy. At necropsy, six weeks later, early rheumatoid changes were evident in both the carpal and tarsal joints. The histopathological changes in the adrenal glands consisted of nearly complete obliterations of the zona glomerulosa and atrophy of the fasciculata and reticularis. The cells were reduced in size, the cytoplasm stained faintly, and the nuclei were small. The changes observed in the kidney were mostly fibrotic. The glomeruli appeared shrunken with encroachment of interstitial connective tissue. Numerous accumulations of round cells were present. Similar accumulations commonly occur in swine kidneys; hence, their significance is not certain.

Proliferation of the synovial villi, round cell infiltration, and beginning subchondral pannus formation in 2 swine receiving the high level of DCA indicated that a disturbance of the normal physiology of the joints had occurred. Selye *et al.*[9, 10] reported that 2 mg. of DCA twice daily in rats had toxic effects when one kidney was removed and a large amount of salt was added to the drinking water. These rats developed periarteritis nodosa, nephroscle-

rosis, and lesions in the heart and blood vessels resembling those of rheumatic fever in man. A high incidence of acute or chronic arthritis developed if the animals were subjected to cold, or if the thyroid or adrenals were removed. Harrison [3] was unable to confirm the role of DCA in causing arthritis, and he believed that infection was intimately concerned with nephrosclerosis and the production of rheumatic cardiac lesions.

In the present experiments, high DCA dosage apparently produced arthritis in normal shoats. The systemic, ill-effects of DCA in normal swine were largely inhibited by the concomitant administration of cortisone. However, at the dosage used, this glucocorticoid failed to prevent early rheumatoid changes in the joints. When experimentally produced cases of arthritis were treated with DCA, the pathological course of the disease was markedly augmented.

It is difficult to explain the fact that the symptoms were relieved in the arthritic animals receiving DCA and cortisone, whereas in normal swine the same level of cortisone failed to prevent the arthritic changes which were produced by DCA. Possibly, the beneficial effects of combined steroid therapy in the former case reflected a greater need for glucocorticoid, since adrenal pathological changes had occurred prior to hormonal therapy. It would appear logical that exogenous glucocorticoid would have a greater inhibiting effect on the pituitary-adrenal axis in the normal animal than it would in the animal with impaired adrenal function. It is apparent that the mineralocorticoids or the mineralocorticoid:glucocorticoid ratio may be concerned in the development of advanced chronic arthritis in swine, particularly since no infectious agent could be isolated from the joints 226 days after exposure.[11]

SUMMARY

1) In swine experimentally infected with *Erysipelothrix rhusiopathiae*, a chronic proliferative polyarthritis developed. Degenerative changes in the adrenal glands and fibrotic lesions in the kidneys were found in animals with advanced cases of chronic arthritis.

2) Vegetative endocarditis was produced in 3 of 4 animals by repeated intravenous injections of cultures of *Ery. rhusiopathiae*.

3) The subcutaneous injection of 1 Gm. of desoxycorticosterone acetate (DCA), suspended in 20 cc. of sesame oil, twice weekly, caused death in 2 shoats, while those receiving 500-mg. doses were affected but survived. Two shoats which each received 2 Gm. of DCA weekly plus 75 mg. of cortisone daily developed acute arthritis.

4) The subcutaneous injection of 1 Gm. of DCA twice weekly in 6 of 7 shoats with acute arthritis augmented the pathological course of the disease.

5) Two shoats with acute polyarthritis due to *Ery. rhusiopathiae* showed remission of symptoms and marked clinical improvement when given 1 Gm. of DCA twice weekly, plus 75 mg. cortisone daily over an eight-week period. Also, 2 other shoats, similarly affected, responded favorably to 500 mg. of DCA twice per week plus 50 mg. of cortisone daily. The untreated control animal died.

6) The relationship of the adrenal and kidney pathological changes and the development of arthritis in swine is discussed.

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Propagation of Vesicular Exanthema Virus on Xenoplastic Grafts

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THE HIGH COST and type of facilities required for the propagation of vesicular exanthema (VE) virus has stimulated an interest in the propagation by means other than swine. The artificial cultivation of VE virus has been previously reported in embryonic pigskin fragments embedded in plasma clots and chopped whole embryos suspended in Baker's solution.[1,2] This report describes still another procedure which was found to be successful for propagation of four immunologically distinct types of VE virus (A, B, C, and D). The technique employed closely parallels that described in the studies of herpes simplex and vaccinia viruses.[3]

MATERIALS AND METHODS

Virus.—Immunotypes A, B, C, and D of VE virus were used. The initial inoculums for each trial consisted of a 10.0 per cent suspension, by weight, of the coverings of vesicles of the snout and feet of infected swine. The suspension was prepared in 0.9 per cent buffered saline and clarified with Lloyd's reagent as described for the preparation of antigens for the complement-fixation test.[4] The inoculums were treated with 5,000 units of penicillin and 2,500 μg. of streptomycin per milliliter and incubated at 6 C. for at least two hours prior to use.

Tissues.—Gravid uteri from eastern market sows were obtained from a slaughterhouse. Embryos, ranging from 3 to 12 cm. long, as determined by external palpation of the uterus, were selected. The embryos were removed aseptically and placed in a sterile pan. The skin of the embryo was removed by inserting a pair of blunt forceps under the skin at the base of the head and forcing the instrument posteriorly to the tail. Using sterile gloves, the thumbs were inserted into the opening and the skin was peeled from the subcutis and underlying tissues. This technique was found to be rapid, simple, and yielded a harvest of skin with the least amount of connective tissue from the entire body. The skin was cut into plaques approximately 4 cm. in diameter and the pieces stored in cold Simm-Sanders z solution as previously described.[2]

Technique.—The pigskin grafts were prepared by removing plaques of skin from the z solution and placing on a sterile cork. The tissue was stretched and the attached corium was cut into pieces approximately 5 mm. square. While still on the cork, the tissue was flooded with 0.9 per cent buffered saline solution (pH 7.2) containing 500 units of penicillin and 100 μg. of streptomycin per milliliter. The tissues were kept moist with the antibiotic solution while the eggs were being prepared for inoculation. Embryonating chicken eggs, 6 to 10 days old, were prepared by making an artificial air cell to expose the chorioallantoic membrane (c.a.m.). One to three pieces of tissue were placed upon the c.a.m. and the opening in the shell sealed with Scotch tape. The eggs were allowed to incubate at 37 C. for 24, 48, or 96 hours following the transplantation.

The original virus inoculum was 0.1 ml. of a 10.0 per cent suspension of infected swine vesicles per egg. The inoculation was accomplished by cutting through the Scotch tape with a scalpel and injecting the inoculum with a 1.0-ml. tuberculin syringe. Scotch tape was used to reseal the window and the eggs were incubated for an additional 24 or 48 hours.

The virus was harvested by swabbing the eggs with 70 per cent ethyl alcohol, removing the Scotch tape, enlarging the window, and cutting the c.a.m. with the grafts free of the embryo with iris scissors. The tissue was ground in a mortar with alundum, diluted to ten times its volume with buffered saline and centrifuged at 2,500 r.p.m. for ten minutes. The supernatant fluid was employed for serial passage in xenoplastic grafts on embryonating chicken eggs, for infectivity tests in swine, and for the complement-fixation test. Bovine blood

TABLE 1—Reaction of Swine to Inoculation with Four Immunotypes of Vesicular Exanthema Virus Propagated on Pigskin Grafted to the Chorioallantoic Membrane of Embryonating Chicken Eggs

Immunotype virus	Passages	Preinoculation time of graft (hr.)	Postinoculation period (hr.)	Serial passages inoculated IDS* into hogs producing typical vesiculation
A	1 to 4	24	24	3
A	5 to 8	0	24	8
B	2 to 33	48	24	5, 9, 15, 25, 30
B	34 to 67	96	48	40, 50, 52, 55, 67
C	7	48	24	7
D	11	48	24	5, 11

* IDS = intradermally into the snout.

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agar plates and Sabouraud's yeast medium were employed for detection of bacterial and mycotic contamination.

RESULTS

Four known immunotypes (A, B, C, and D) of VE virus were successfully cultivated on the skin of embryonic pigs grafted onto the c.a.m. of embryonating chicken eggs. Type B was carried through 67 serial passages, while types A, C, and D were carried through eight, seven, and 11 serial passages, respectively. Type B virus was the one chosen for most of the work, whereas the other three types were discontinued once it was evident that the virus had propagated.

The period of incubation prior to inoculation with virus did not appear to be too critical since type A VE virus was serially passed eight times when the grafting period prior to inoculation was 24 hours or less (table 1). A preinoculation period of 24 hours and a postinoculation period of an additional 24 hours did not appear to be sufficient to attain a high degree of grafting, as the pigskin could be generally lifted without difficulty from the c.a.m. When the preinoculation period was extended to 48 to 96 hours, the pigskin adhered to the embryo membrane and required incision for its separation. A 48-hour preinoculation period in combination with a 24-hour postinoculation period appeared to be more desirable, because extension of the grafting and postinoculation periods resulted in some peripheral necrosis of the graft which could be observed macroscopically.

The allantoic fluid carefully removed through an opening away from the pigskin grafts of infected embryonating eggs proved infectious for susceptible swine as long as 96 hours following egg inoculation. This would indicate that the virus had found its way into the allantoic fluid from the source of multiplication.

In three separate trials, attempts were made to propagate VE virus in embryonating chicken eggs in the absence of embryonic swine tissue. The inoculums of the ninth, thirty-first, and fiftieth passages of type B VE virus were serially passed in parallel with the pigskin graft series. The eggs were inoculated on the c.a.m. with 0.1 ml. of the inoculum. After 24 hours' incubation, the c.a.m. was harvested, prepared as a 50 per cent suspension and serial passages continued. The inoculums prepared of the second to the fifth passages were innocuous in all three instances when inoculated intradermally into the snouts of susceptible swine.

Histology.—Microscopically, the successful establishment of the graft to the c.a.m. could be demonstrated by the merging of the two tissues and the presence of nucle-

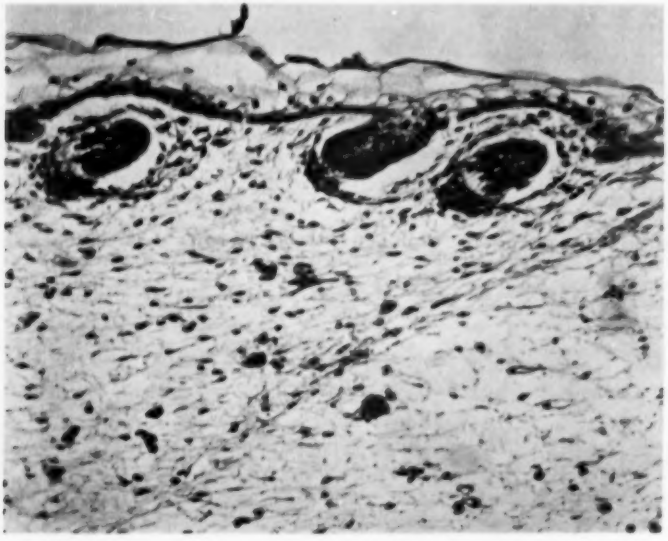

Fig. 1—A 72-hour graft showing the merging of tissues of the chorioallantoic membrane of a 10-day-old embryonating chicken egg and embryonic skin tissue of swine. x 170.

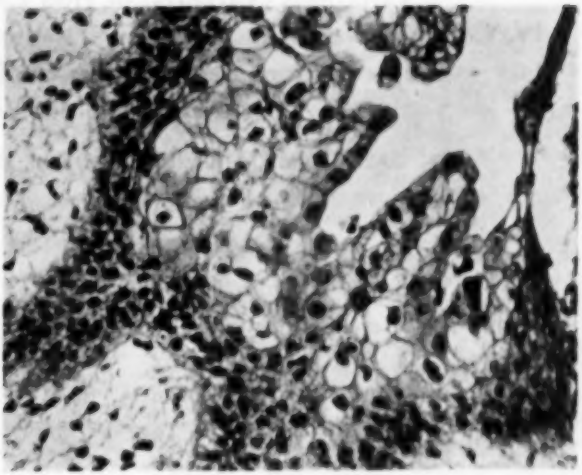

Fig. 2—Hydropic degeneration of epithelial cells in the epidermis of an infected swine skin graft. x 300.

ated avian erythrocytes in the established blood vessels of the porcine corium (fig. 1).

Histological sections of grafted swine skin on the c.a.m. inoculated with type B VE virus (passage 51) 48 hours following transplantation and harvested 24, 48, and 72 hours later, respectively, did not present consistent nor extensive cytopathological lesions when compared with control grafts which were inoculated with buffered saline. Some of the sections, however, showed areas of initial hydropic degeneration (fig. 2). Suspensions prepared from grafts of the passage, when inoculated into swine, proved to be infectious.

Serology.—Harvested grafts picked at random from 22 passages in the series with the four types of VE virus showed no evidence of complement fixation with their homologous swine anti-VE serums. Facilities and cost prohibited titration of the virus in swine. Based upon previous studies, the inability of infectious suspensions to fix complement in the presence of immune serums indicated that the propagation of the virus by this method was minimal or did not yield as much virus as can be expected from vesicles harvested from living swine following inoculation. This can be further substantiated by the fact that vesicles harvested from hogs inoculated with the egg-propagated virus resulted in excellent complement-fixation reactions.

SUMMARY

A method for the propagation of four immunotypes of vesicular exanthema (VE) virus by xenoplastic grafting of embryonic pigskin to the chorioallantoic membrane of embryonating chicken eggs has been described. Type B VE virus was carried through 67 serial passages, whereas types A, C, and D VE virus were passed eight, seven, and 11 times, respectively.

Sufficient virus was recovered from skin graft suspensions and the allantoic fluid of the inoculated eggs to produce extensive and generalized VE when inoculated into susceptible swine, but the virus concentration was not satisfactory as an antigen for the direct complement-fixation test.

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Variations (Variants) of Hog Cholera Virus
IV. Comparative Potencies of Crystal-Violet Glycerol Vaccines Prepared from Regular Hog Cholera Virus and Variant Hog Cholera Virus

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IN THE REPORT of investigations by Dale *et al.*[1] of losses of hogs in farm herds in 1949 and 1950 following treatment with the simultaneous serum-virus method of immunization, it was determined that these losses were due to the use of a virus with variant characteristics. This conclusion was based partly upon results of experiments, reported in the above publication, using hog cholera viruses and anti-hog cholera serums. Additional evidence that this particular virus used in the field possessed variant characteristics was obtained from experiments in which crystal-violet glycerol (CVG) vaccines prepared from it and regular viruses were used to immunize pigs, with subsequent cross challenge of the immunity. These experiments were started just prior to the publication of U. S. Department of Agriculture news release 2396-49, dated Nov. 8, 1949. This paper presents the results obtained from some of the tests of such CVG vaccines.

COMPARATIVE TESTS OF POTENCIES OF CRYSTAL-VIOLET GLYCEROL VACCINES PREPARED FROM REGULAR AND VARIANT VIRUS

MATERIALS

1) BAI Virus 286.—This was a regular, unphenolized BAI virus prepared at Ames.[1] The minimum lethal dose (m.l.d.) of this virus was 1/5,000,000 cc. when prepared and 1/1,000,000 cc. 226 days and also 331 days after preparation. It had been stored at —40 F., or below, for 264 and 726 days when it was used in experiments 1 and 2, respectively. The m.l.d. of the challenging viruses used in these experiments was determined at the Hog Cholera Research Station, Ames, Iowa, according to the method used by Cole *et al.*[2]

2) Variant Virus A Used as the Source of Other Variant Virus.—This commercial hog cholera virus, described by Dale *et al.*,[1] was associated with the postvaccination losses in farm herds in 1949. Its m.l.d. was not less than 1/8,000 cc. Both pigs injected with 1-cc. amounts of the virus died from hog cholera. Neither of the 2 pigs injected with 1/8,000 cc., the next lower amount tested, nor any of the pigs injected with still smaller amounts were affected. The surviving pigs proved to be susceptible when challenged later with hog cholera virus. Additional information on this virus may be found in the above reference.

3) Variant Virus 8014.—This was a proved variant virus representing the ninth serial, simultaneous passage of virus A and serum. A titration of this virus showed that its m.l.d. was at least 1/10,000 cc. but not as low as 1/100,000 cc. When variant virus 8014 was tested[*] for variant characteristics, 5 cc. of BAI experimental serum 1 counteracted it in 2 of 6 pigs and 15 cc. of BAI experimental serum 1 counteracted it in 3 of 4 pigs. This might suggest that the variant characteristics of variant virus 8014 were not marked. This virus had been stored at —40 F., or below, for 75 days when it was used in experiment 1.

4) "Variant" Virus 8125.—This virus was prepared by pooling the defibrinated blood of 3 pigs challenged for cholera susceptibility after remaining normal in a virus titration test. For the purpose of such challenge, they were injected with variant virus 8100, which was the second serial passage of variant virus A passed without serum. Variant virus 8125, therefore, represented the third serial passage of virus A without serum, and is called variant virus, although it was not tested for variant characteristics or titer. It had been stored since preparation at —40 F., or below, for 263 days, when it was used in experiment 1.

5) Variant Virus 9096 Used in Experiment 2.—This was the seventh serial passage of variant virus s.n. 2 without serum. Variant virus s.n. 2 was the ninth passage of virus A with serum.[*] A preliminary test[2] of variant virus 9096 indicated that this virus had variant characteristics in that 2 pigs treated simultaneously with it and 15 cc.

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[*] For further details of description and derivation of variant virus s.n. 2, see reference 4.

of BAI experimental serum 1 sickened and were inadequately protected, while 2 pigs treated simultaneously with regular BAI virus 286 and 15 cc. of the same serum did not sicken. The variant characteristics of variant virus 9096 were confirmed in a further test in which it was compared with the first passage (virus 8652) of a proved variant virus, virus H.† The test of virus 9096 and virus 8652 gave similar results[8] in that all pigs treated simultaneously with the viruses and 15 cc. of BAI experimental serum 1 died and all pigs treated with 30 cc. of the same serum lived. The m.l.d. of variant virus 9096 was between 1/500,000 cc. and 1/1,000,000 cc. One of 2 pigs injected with the smaller dose and 1 of 2 pigs injected with the larger dose survived. This virus had been stored since preparation at —40 F., or below, for 147 days when used in experiment 2.‡

6) Crystal-Violet Glycerol Vaccine 96.—This was a regular BAI CVG vaccine prepared from a regular virus, as routinely produced at Ames, and was a mixture of 79 sublots, each of which was prepared from defibrinated blood of an individual pig.

7) Crystal-Violet Glycerol Vaccine 8014.—This vaccine was prepared from variant virus 8014. It was tested previously and had failed to protect pigs either in 5-cc. or 10-cc. doses against exposure with regular BAI virus 286. However, it was included in this test in order to determine whether it would protect against the virus from which it was prepared or against the variant virus 8125.

8) Crystal-Violet Glycerol Vaccine 8125.—This vaccine was prepared from the pooled blood of the same 3 pigs which made up the variant virus 8125 described above.

9) Crystal-Violet Glycerol Vaccine 9096.—This vaccine was prepared from variant virus 9096.

10) BAI Experimental Serum 1.—On the basis of exhaustive tests[1] this serum was selected to detect variant virus. If the unknown virus to be tested had variant characteristics, pigs injected with 2 cc. simultaneously with 15 cc. of BAI experimental serum 1 would not be adequately protected, whereas the contrary would be true if the test material was regular virus.

Experimental Pigs.—The 102 pigs used in experiment 1 were obtained from the U. S. Department of Agriculture swine-breeding herd at Beltsville, Md. Previous work has indicated differences in pigs in their response to immunization, some pigs developing a higher degree of immunity than others. It was, therefore, desirable to use litter mates as representatives in the various groups receiving the different treatments. The pigs obtained had complete histories, especially with reference to the susceptibility of the dams and the pigs and the occurrence of disease on the premises. More than 200 pigs were delivered and held in isolation. They were delivered on the day of weaning when 56 days old. All were obtained within a period of 17 days. About ten days after weaning, all the pigs were treated for worms.

A good percentage of this group of pigs had made less than normal gains in weight. A few of the poorest were culled for necropsy. Most of them showed consolidated areas of the anterior lobes of the lungs. This pneumonic condition may have accounted for febrile reactions, which were recorded as occurring in a limited portion of the 200 pigs prior to the time of exposure. Several pigs, including 1 unvaccinated and 4 vaccinated pigs assigned to the control group, died before exposure. Hog cholera was excluded by animal-inoculation tests and by failure of the condition to spread to susceptible pigs in the same pen. The pigs were vaccinated in their isolation quarters and, 21 days later, were moved to the exposure barns. The distance between the isolation quarters and the exposure barns was about 3 miles.

Sixty-six pigs were used in experiment 2. The pigs were acquired in the same manner and under the same arrangements as the 102 pigs used in experiment 1. They were also kept in isolation after vaccination and moved to the Animal Disease Station for the challenge exposure. No febrile or other abnormal condition was observed in these pigs prior to exposure.

METHODS

Hemocultures on broth and agar slants were made for each blood specimen, and necropsies were performed on all pigs which died or were killed when moribund or as survivors of the experiment. Cultures for bacterial growth on agar slants were made of the heart blood, spleen, kidney, liver, and lung of the pigs which died or were killed when moribund. Similar bacteriological examinations were made of the heart blood, spleen, and lung of the survivors. Challenge exposures were made three weeks after vaccination by subcutaneous injections of 2 cc. of virus. Bacteriological examination indicated that all viruses were free of bacterial agents.

Procedure Used for the Production of Crystal-Violet Glycerol Vaccines.—As stated above, CVG vaccine 96 was produced from the pooled sublots of vaccines prepared from defibrinated blood of 79 pigs, each of which had been injected with regular hog cholera virus.

Crystal-violet glycerol vaccine 8014, CVG vaccine 8125, and CVG vaccine 9096 were prepared at the Animal Disease Station at Beltsville, Md. These experimental vaccines were prepared according to the method described by Cole and Henley.[5] Vaccines 8014 and 9096 were each prepared from defibrinated blood from a single pig. A portion of

† The tests demonstrating the variant characteristics of virus H were reported by Dale *et al.*[1] In that report, it was shown that, at different places and at different times and with pigs of different weights, BAI experimental serum 1 in doses as high as 30 cc. failed to protect pigs against virus H, while the same serum in doses as small as 5 cc. protected pigs against regular BAI virus 286.

‡ For further details of derivation and description, see reference 3.

this blood of each pig was stored at —40 F., or below, in order to have a strictly homologous virus with which to challenge pigs vaccinated with the vaccines. A third portion of the defibrinated blood from pig 8014 was used to prepare a hyperimmune serum. No hyperimmune serum was prepared with 8125 or 9096 viruses.

Method Used for Evaluating Potency of Vaccines.—A system of points assigned to various symptoms was used in determining whether a pig was adequately or inadequately protected. Pigs were fed once a day and closely observed at that time for degree of appetite. A fair appetite was given a value of 1, a slight appetite a value of 2, and no appetite a value of 3. An animal that had a total of ten points or less during the three-week observation period after exposure was considered to be adequately protected, although consideration was also given to the degree and length of febrile reactions. Animals that had a total of 11 points or more during this period were considered to be inadequately protected.

RESULTS

In experiment 1, the 102 pigs from 26 litters were distributed among the 21 treatment groups. The details of the vaccinations, challenge exposures, and the results are shown (table 1).

In experiment 2, the 66 pigs from ten litters were distributed among the 14 treatment groups. The details of the vaccinations, challenge exposures, and the results are shown (table 2).

DISCUSSION

In experiments 1 and 2, there was evidence that pigs vaccinated with a CVG vaccine prepared from regular hog cholera virus were protected when challenged with a regular hog cholera virus, but like pigs similarly vaccinated with the same dosage of the same CVG vaccine were not as well protected against a challenge of variant hog cholera virus. This lesser protection against variant virus occurred even though the infectivity titer of the variant virus was at least ten to 100 times less than that of the regular virus. This difference in immunity was like that which has been demonstrated in some other virus diseases wherein animals treated with a vaccine prepared from one strain of virus possessed varying degrees of immunity against different strains of virus. In foot-and-mouth disease, variants within types of virus are common, especially in type A.

In experiment 1, the 6 pigs vaccinated

TABLE 1—Results in Experiment 1 of Cross-Immunity Tests of Pigs Vaccinated with Crystal-Violet Glycerol (CVG) Vaccines Prepared from Regular and Variant Hog Cholera Viruses

Vaccines				Viruses			Results				
No. of pigs	CVG vaccine 96 (regular)*	CVG vaccine 8014 (variant)*	CVG vaccine 8125 ("variant")*	Regular BAI virus 286*	Variant virus 8014*	"Variant" virus 8125*	N†	Sl	Sv	D	Adequately protected (%)
2	5 cc.	.....	.....	2 cc.	.....	.....	2	0	0	0	100.0
8‡	2 cc.	.....	.....	2 cc.	.....	.....	6	1	0	0	100.0
2	5 cc.	.....	.....	.....	2 cc.	.....	2	0	0	0	100.0
8§	2 cc.	.....	.....	.....	2 cc.	.....	0	1	3	3	14.0
2	5 cc.	.....	.....	.....	.....	2 cc.	2	0	0	0	100.0
8§	2 cc.	.....	.....	.....	.....	2 cc.	2	3	1	1	71.0
2	.....	5 cc.	.....	2 cc.	.....	.....	0	0	0	2	0
8§	.....	2 cc.	.....	2 cc.	.....	.....	0	0	0	7	0
2	.....	5 cc.	.....	.....	2 cc.	.....	0	0	0	2	0
8	.....	2 cc.	.....	.....	2 cc.	.....	0	0	1	7	0
2	.....	5 cc.	.....	.....	.....	2 cc.	0	0	0	2	0
8	.....	2 cc.	.....	.....	.....	2 cc.	0	0	0	8	0
2	.....	.....	5 cc.	2 cc.	.....	.....	0	2	0	0	100.0
8	.....	.....	2 cc.	2 cc.	.....	.....	4	1	0	3	62.5
2	.....	.....	5 cc.	.....	2 cc.	.....	0	0	1	1	0
8	.....	.....	2 cc.	.....	2 cc.	.....	0	0	2	6	0
2	.....	.....	5 cc.	.....	.....	2 cc.	0	0	0	2	0
8§	.....	.....	2 cc.	.....	.....	2 cc.	0	2	0	5	28.6
4§	.....	.....	.....	2 cc.	.....	.....	0	0	0	3	0
4	.....	.....	.....	.....	2 cc.	.....	0	0	0	4	0
4	.....	.....	.....	.....	.....	2 cc.	0	0	0	4	0

Necropsy.—All pigs which died or were killed when moribund had lesions commonly associated with hog cholera.
Bacteriology.—*Salmonella enteritidis* was obtained on agar slant cultures of the tissues from only 5 pigs. *Salmonella typhimurium* was obtained from 1 pig. The 6 pigs from which they were obtained were pigs that died. *Pasteurella multocida* was obtained from 23 pigs that died and 3 pigs that were killed at the termination of the test.

* For description and derivation of these products, see "Materials" in text. † N = normal, Sl = slight reaction, Sv = severe reaction, and D = died or killed when moribund; pigs were considered adequately protected if they remained normal or did not have more than a slight reaction as a result of the challenge exposure. ‡ One pig was eliminated from the test after vaccination when it developed a debilitating condition. § One pig in this group died before the challenge exposure; the lesions at necropsy were not typical of hog cholera.

with 5-cc. doses (the recommended field dosage) of CVG vaccine 96 were adequately protected regardless of whether the challenge virus was a regular virus or a variant virus. However, when vaccination doses of 2 cc. were used, only the pigs challenged with regular virus were adequately protected. Of 7 pigs thus treated, all responded favorably and 6 of them showed no impairment of appetite. In contrast to this, of the 7 pigs vaccinated with 2 cc. of regular CVG vaccine 96 and exposed with variant virus 8014, 3 died, 3 had severe reactions, and 1 had a slight reaction. Thus, only 14 per cent were adequately protected. Of the 7 vaccinated with 2 cc. of CVG vaccine 96 and exposed with variant virus 8125, 1 died, 1 had a severe reaction, 3 had slight reactions, and 2 remained normal. Thus, 71 per cent were adequately protected.

Crystal-violet glycerol vaccine 8014 failed to produce adequate immunity in any of the pigs regardless of which virus was used for challenge. All pigs sickened and all except 1 died before the twenty-first day after the challenge exposure. The only difference observed was that most of the pigs challenged with variant virus 8014 survived five or six days longer than those challenged with regular BAI virus 286 or the variant virus 8125. Since the m.l.d. titer of the virus used for the preparation of the vaccine was 1/10,000 cc., it is possible that there was insufficient virus to produce a satisfactory vaccine.

Of the 6 pigs vaccinated with 5-cc. doses of CVG vaccine 8125, only the 2 which were challenged with regular BAI virus 286 were adequately protected; both had slight reactions. Of the 2 pigs challenged with variant virus 8014, 1 died on the thirteenth day and the other had a severe reaction. The latter pig was apparently normal on the eighteenth day after exposure, although it had been off feed eight of the 18 days. The 2 pigs exposed with the variant virus 8125 died, 1 on the tenth day and 1 on the eleventh day. In this case, a CVG vaccine used in recommended dosage produced adequate immunity when challenged with one virus (regular BAI virus 286) but failed to produce appreciable immunity against either of two other viruses, one a proved variant (variant virus 8014) and the other a passage of a variant virus

TABLE 2—Results in Experiment 2 of Cross-Immunity Tests of Pigs Vaccinated with Crystal-Violet Glycerol (CVG) Vaccines Prepared from Regular and Variant Hog Cholera Viruses

No. of pigs	Vaccines: CVG vaccine 96 (regular)*	Vaccines: CVG vaccine 9096 (variant)*	Viruses: Regular BAI virus 286*	Viruses: Variant virus 9096*	Dose of BAI exptl. serum 1*	Results: N†	Sl	Sv	D	Adequately protected (%)	Bacteriology: Organisms‡ and No. of pigs (in parentheses) in which found
10	5 cc.	.....	2 cc.	.....	.....	7	3	0	0	100	Past. (2).
10	5 cc.	.....	.....	2 cc.	.....	1	6	0	3	70	S.ch. (2); Coryn. (2); Ps.py. (1); Past (1).
10	.....	5 cc.	2 cc.	.....	.....	0	1	1	8	10	Prot. (4); Ps.py. (4); Coryn. (4); Past. (3); S.ch. (1).
10	.....	5 cc.	.....	2 cc.	.....	1	2	2	5	30	S.ch. (3); Past. (2).
2	.....	.....	2 cc.	.....	5 cc.	1	0	0	1	50	Past. (2).
2	.....	.....	.....	2 cc.	5 cc.	0	0	0	2	0	S.ch. (1); Past. (1); Ps.py. (1).
2	.....	.....	2 cc.	.....	15 cc.	2	0	0	0	100	.....
2	.....	.....	.....	2 cc.	15 cc.	0	0	0	2	0	Past. (2); S.ch. (1).
2	.....	.....	2 cc.	.....	30 cc.	1	0	1§	0	50	Past. (1).
2	.....	.....	.....	2 cc.	30 cc.	0	0	1	1	0	Coryn. (1).
2	.....	.....	.....	2 cc.	45 cc.	0	1	1	0	50	.....
2	.....	.....	.....	2 cc.	60 cc.	0	2	0	0	100	.....
5	.....	.....	2 cc.	.....	.....	0	0	0	5	.....	S.ch. (2); Past. (1); Ps.py. (1).
5	.....	.....	.....	2 cc.	.....	0	0	0	5	.....	Coryn. (2); Past. (1).

Necropsy.—All pigs which died or were killed when moribund had lesions commonly associated with hog cholera.
* For description and derivation of these products, see "Materials" in text. † N = normal, Sl = slight reaction, Sv = severe reaction, and D = died or killed when moribund; pigs were considered adequately protected if they remained normal or did not have more than a slight reaction. ‡ S.ch. = *Salmonella choleraesuis*, Past. = *Pasteurella multocida*, Prot. = Proteus sp., Ps.py. = *Pseudomonas pyocyanea*, and Coryn. = Corynebacterium sp.; the number of isolations do not balance with the number of pigs in a particular treatment in some cases because more than one organism was isolated from a single pig—*S. choleraesuis* was obtained only from pigs which died and *Past. multocida* was obtained from 10 pigs which died and from 5 pigs which were killed at the termination of the test. § This pig was normal for 12 days; it then became crippled and had a febrile reaction and impaired appetite for a week before recovery.

which was strictly homologous to the virus used to prepare the vaccine.

The results obtained with the 2-cc. doses of the CVG vaccine 8125 are in conformity with the results obtained with the 5-cc. doses. Of the 8 pigs vaccinated with 2-cc. doses and challenged with regular BAI virus 286, 4 remained normal, 1 had a slight reaction, and 3 died, the percentage having adequate protection being 62.5 per cent. Of the 8 pigs vaccinated with 2-cc. doses and challenged with variant virus 8014, 6 died, 2 had severe reactions but had apparently recovered on the 18th day; none were adequately protected. Of the 7 pigs vaccinated with 2-cc. doses and challenged with variant virus 8125, 5 died and 2 had slight reactions. Only 28.6 per cent were adequately protected. Thus, similar to the results obtained with the 8014 vaccine, the CVG vaccine 8125 when used in 2-cc. amounts failed to produce adequate protection against any of the three viruses. The vaccine, however, gave adequate protection when used in doses of 5 cc. against challenge with the regular virus but not against variant virus 8014 or homologous virus. The results suggest the need of having a titration of the virus to exclude the possibility that insufficient virus may be the factor responsible for the impotency of the vaccine. It would seem that the amount of variant virus required for a satisfactory vaccine should be of the order of that which is generally satisfactory with the regular virus.

In experiment 2, the 10 pigs vaccinated with regular CVG vaccine in 5-cc. amounts, the dose recommended for field use, were adequately protected against a challenge with regular hog cholera virus. However, like pigs similarly vaccinated with the same dose of the same vaccine were not adequately protected against variant virus—3 of 10 dying of hog cholera when thus exposed.

As in experiment 1, pigs vaccinated with vaccine prepared from variant virus were not adequately protected from challenge with homologous variant virus or when challenged with regular virus. Of the 10 pigs vaccinated with the variant vaccine, only 1 was adequately protected when challenged with regular virus and, of the 10 vaccinated with the same vaccine and challenged with variant virus, only 3 were adequately protected. The m.l.d. titer of the virus used for the production of this vaccine was relatively low, thus suggesting a possible explanation for the low potency.

The results shown in table 2, of the serological test of the variant virus 9096 used in this experiment, indicated that this virus had marked variant characteristics. Doses of 5 cc., 15 cc., and 30 cc. of BAI experimental serum 1 failed to counteract this virus. In 45-cc. amounts it counteracted the virus in 1 of 2 pigs. Both pigs that received 60 cc. of serum were protected. In contrast, when regular virus 286 was injected with the same doses of serum, all pigs were adequately protected that had received 15 cc. or more of serum, as well as 1 of 2 pigs that received only 5 cc. of serum. One of the pigs injected with 30 cc. was normal for 12 days. It then became crippled, but recovered after seven days' illness.

It would seem that one method of approach to the problem of producing a CVG vaccine which may provide adequate protection against variant virus is to use virus with an m.l.d. titer as high as that of the regular virus used for the production of regular CVG vaccines. Such virus may be obtained by proper serial passage of variant virus. The limited number of serial passages used so far have not proved satisfactory for obtaining yields with high titers.

Over the years, close attention has been given by various investigators to the role played by *Salmonella choleraesuis* and *Pasteurella multocida*, not only as the primary causes of specific disease entities but also as secondary agents associated with viral diseases and especially hog cholera. Studies directed specifically to this line of investigation have been carried on not only throughout these and several previous reports but also with the original field investigations. Only a review of such bacteriological studies will permit proper evaluations of results in most experiments dealing with comparative tests of regular and variant viruses.

Failure to obtain *S. choleraesuis* in any instance in experiment 1 (table 1) justifies the elimination of this microorganism as a factor in the final results.

While *S. choleraesuis* was obtained from 10 pigs in experiment 2 (table 2) it was not obtained from any of the 10 pigs vaccinated with regular CVG vaccine and

challenged with regular virus. Seven of the latter 10 pigs had no impaired appetite and only 1 of the 7 had a febrile reaction. The impaired appetite of 2 of the 3 so affected consisted of a slightly reduced appetite on one day. One of the 2 had a febrile reaction of 104.4 F. on one day. The third pig had slight, fair, and fair appetites on days 9, 10, and 11, respectively. During this period, it had a febrile reaction which varied from 105.0 to 107.2 F. The outcome in this group of pigs is another demonstration where *S. choleraesuis* was not incriminated as a complicating factor.

The 10 pigs in experiment 2 from which *S. choleraesuis* was obtained consisted of 8 vaccinated pigs and 2 controls. All were pigs that died. It is with such results in vaccinated pigs that it is not always, if at all, possible to determine routinely by means presently available whether death was due to hog cholera or a primary or secondary *S. choleraesuis* infection. By using sufficient numbers of pigs in the different groups, as in this experiment, it may be possible to eliminate from the calculations, if necessary, pigs from which *S. choleraesuis* is obtained and still have statistically significant data from the remainder.

Thus, in the case where *S. choleraesuis* was obtained from 2 of the 3 pigs that died following treatment with regular vaccine 96 and subsequent challenge with variant virus 9096, an evaluation other than that used in the text might limit death caused by hog cholera to 1 pig with the cause of death of the other 2 undetermined. Similar application may be given to findings of this nature with *Past. multocida*.

The results in experiment 1 did not furnish information which lent itself to comparison between litters or litter mates of the degree of susceptibility to hog cholera or the degree of immunization response to vaccine. This was due to the relatively low immunizing potency of vaccines 8014 and 8125 and to the 100 per cent protection of pigs vaccinated with 2-cc. doses of vaccine 96 and challenged with regular virus 286. However, each pig from seven litters in experiment 1 vaccinated with 2 cc. of regular vaccine, and challenged with regular virus, was adequately protected despite any low degree of immunity response which any of these 7 pigs may have possessed. Likewise, each pig from 10 litters in experiment 2 similarly treated but with a 5-cc. dose of vaccine was adequately protected despite any low level of immunization response if present.

SUMMARY

Three hog cholera crystal-violet glycerol (CVG) vaccines were each tested for immunogenicity in one experiment against three different viruses. In another experiment, one vaccine from the previous experiment was retested and another vaccine was tested for potency against two viruses.

The viruses were (1) regular BAI virus 286; (2) variant virus 8014, the ninth serial, simultaneous passage of variant virus and serum; (3) "variant" virus 8125, the third serial passage of variant virus without serum; and (4) variant virus 9096, the seventh serial passage without serum of a variant virus following nine passages simultaneously with serum.

The vaccines were (1) CVG vaccine 96, a vaccine prepared from a regular BAI virus; (2) CVG vaccine 8014 (variant), a vaccine prepared from the virus representing the ninth serial, simultaneous passage of variant virus and serum; (3) CVG vaccine 8125 ("variant"), a vaccine prepared from the third serial passage of variant virus without serum; and (4) CVG vaccine 9096 (variant), a vaccine prepared from the virus representing the seventh serial passage without serum of a variant virus following nine serial passages simultaneously with serum.

The pigs were challenged three weeks after vaccination.

Crystal-violet glycerol vaccine 96 in 5-cc. doses adequately protected all of 16 pigs (100 %) when challenged by regular BAI virus 286, variant virus 8014, or variant virus 8125 and adequately protected 7 of 10 pigs (70.0 %) when challenged by variant virus 9096. In 2-cc. doses, CVG vaccine 96 adequately protected all of 7 pigs (100 %), 1 of 7 pigs (14.0 %), and 5 of 7 pigs (71.0 %) when challenged by regular BAI virus 286, variant virus 8014, and variant virus 8125, respectively.

Crystal-violet glycerol vaccine 8014 (variant) gave no appreciable protection in either 5-cc. or 2-cc. doses against regular BAI virus 286, variant virus 8014, or variant virus 8125.

Crystal-violet glycerol vaccine 8125

(variant) in 5-cc. doses adequately protected 2 pigs (100 %) and in 2-cc. doses adequately protected 5 of 8 pigs (62.5 %) when challenged by regular BAI virus 286. It gave no appreciable protection in 5-cc. doses against variant virus 8014 or variant virus 8125. In 2-cc. doses, it failed to protect any of the animals against variant virus 8014 but adequately protected 2 of 7 pigs (28.6 %) against variant virus 8125.

Crystal-violet glycerol vaccine 9096 (variant) in 5-cc. doses adequately protected 1 of 10 pigs (10.0 %) when challenged by regular BAI virus 286 and 3 of 10 pigs (30.0 %) when challenged by variant virus 9096.

CONCLUSIONS

1) Hog cholera crystal-violet glycerol (CVG) vaccines prepared from regular virus may produce less protection against challenge with variant hog cholera virus than against challenge with regular hog cholera virus.

2) High quantitative titers of variant hog cholera virus in defibrinated blood from swine have not been obtained as uniformly as those of regular virus. This may account for difficulties encountered, so far, in producing as potent vaccines from variant virus as from regular virus.

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The Use of Low Level Piperazine on Pigs Experimentally Infected with *Ascaris Lumbricoides*

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THE INTEREST in the class of chemical compounds known as "piperazines" has been intense in the past several years. Most of the experimental and field trials have involved the use of therapeutic doses. This experiment was stimulated by the report of Brown and Chan,[1] indicating that high dosage of piperazine-HCL given orally to mice before and after infection with embryonated eggs of *Ascaris lumbricoides* prevented the hatching larvae from invading the blood stream and lungs. The purpose of this experiment was to try low level feeding a week before, and three weeks after, infecting ascarid-free pigs. The animals would then be killed to determine evidence of migration or infection.

MATERIALS AND METHODS

The drug used was parvex®* (polymeric piperazine-1-carbodithioic acid). According to Leiper,[2] 100 mg. per kilogram of body weight was completely effective against *A. lumbricoides* in individually dosed pigs, although often 125 mg./kg. was used in heavy infections. In this experiment, approximately 10 mg./kg. per day was used. Since the pigs averaged about 35 lb. (16 kg.), each pig was to receive about 160 mg. of the drug per day in the feed. Six- to 8-week-old pigs eat about 2 to 4 lb. dry feed per day so 53 mg. of the drug was incorporated in each pound of dry feed. This was done by a small premix which was added to 100 lb. of feed and mixed for one-half hour in a concrete mixer. A regular pig-growing ration was used.

The pigs used were Chester Whites, 8 weeks old, taken from thoroughly scrubbed sows one day after farrowing and raised in screen-floored cages with purely synthetic diets (as a part of nutritional studies). In the past four years, 15 pigs from this source, used as controls for other ascarid experiments, have never revealed any liver lesions nor were any ascarids ever recovered while screening the contents of the small intestine through 80-mesh per square inch screens. It is believed that these pigs are as near ascarid-free as is possible.

The infective ascarid eggs were obtained by removing the first inch of each uterine horn of ascarids taken from hogs at a packing plant, breaking them up in a small Waring blendor, and then allowing the eggs to embryonate for 60 days in Petri dishes containing 1:500 formalin. At the time of infection, the material in the Petri dishes was washed several times with tap water and suspended for counting. After counting, appropriate doses were placed in 000 gelatin capsules and administered orally to individual pigs. The viability of the ascarid eggs was checked by administering 0.5 cc. of the stock solution of eggs to a rat via a stomach tube. This rat, infected Dec. 29, 1955, died Jan. 2, 1956, and necropsy revealed pneumonia, petechial hemorrhages, and consolidation of the lung lobes. Larvae were found in the lungs.

The principal and control pigs were raised in tile-walled rooms with concrete floors and were fed and watered from sterilized iron troughs. The walls and floors were thoroughly scrubbed with quaternary ammonium disinfectant, washed with hot tap water, and allowed to thoroughly dry before beginning the experiment. Separate sets of boots were maintained outside each room as well as separate galvanized feed cans.

The pigs were placed in 3 groups. In group 1, 4 pigs, a, b, c, and d, were given the piperazine feed for one week, then they received eggs by gelatin capsule in amounts of 17, 34, 85, and 170 thousand, respectively.

In a like manner, in group 2, 3 pigs, e, f, and g, after one week on normal feed, received 34, 85, and 170 thousand eggs, respectively. These pigs were to act as "worm controls" in the experiment.

In group 3, 2 pigs, h and i, received no worm eggs and were maintained on normal feed. They were used to indicate freedom from ascarid infection as well as comparative growth during the experiment.

The results of these experiments are given in table 1.

DISCUSSION

Several things about the data of this experiment should be explained. The difference in weight gains between the controls and the infected (principal) pigs may be explained by the effects of the actual passage of the larvae and the respiratory embarrassment following. Groups 1 and 2 were infected with eggs on December 28 and, as early as January 2, they were

From the Department of Microbiology and Public Health, Michigan State University, East Lansing.

Journal article 1938, Michigan Agricultural Experiment Station, East Lansing.

* Supplied through courtesy of the Upjohn Co., Kalamazoo, Mich.

TABLE 1—Effect of Continuous Piperazine * Feeding on Experimental Ascaris Lumbricoides Infections in Pigs

	Pig	Initial weight (lb.)	Necropsy weight (lb.)	Weight gain (lb.)	Infective egg dose	Liver lesions	Respiratory embarrassment	Worms present
Group 1, drug + worms	a	26	64	38	17,000	+	+	0
	b	34	67	33	34,000	+	+	0
	c	47	75	28	85,000	+	+	0
	d	39	64	25	170,000	+	+	0
Group 2, worms	e	28	61	33	34,000	+	+	0
	f	30	62	32	85,000	+	+	0
	g	35	66	31	170,000	+	+	0
Group 3, controls	h	28	72	44	0	0	0	0
	i	33	74	41	0	0	0	0

* Parvex.

coughing excessively and their food intake was diminished considerably. This probably accounted for their lower weight gain as compared to the controls.

Surprisingly, no young worms were found developing in the small intestine although ascarid lesions were present in the liver. The fate of the developing larvae was not determined; time did not permit histological sections of the lesions. One might reason that the piperazine in the enteron caused the larvae to be flushed out but, in recent experiments,[3] 3-week-old Ascaris larvae were recovered from the small intestine of pigs on one-fifth the therapeutic dose per day of the same piperazine. The larvae in the present experiment were probably in the liver or lungs with some perhaps being excreted undeveloped. The fact that larvae migrated easily in rats indicates that the ascarid eggs would develop to an infective state.

The failure to produce experimental *A. lumbricoides* infections in clean pigs has been reported. Martin,[4] in one experiment with 5 pigs, failed to establish infection but no explanation was given. De Boer [5] had similar experiences with single infections in pigs and indicated that probably one infection was not sufficient to overcome individual resistance even though lung and liver involvement did occur. In one of his experiments, 2 of 7 pigs getting individual doses of infective eggs died ten days after birth but, after 50 days, no worms were found at necropsy of the remaining animals. The cause of death of the 2 pigs was not indicated. De Boer,[5] using multiple infections during a period slightly over two months, was able to produce infection in all of 7 pigs.

Although Brown and Chan [1] were able to inhibit migratory phases of pig ascarids in mice with piperazine-HCL given orally in large doses before and after infection, it does not appear that the piperazine used in this experiment with pigs can successfully inhibit Ascaris infection when administered in the feed at the rate of one-tenth of the therapeutic dose per day throughout the experiment and even when administered one week prior to infection.

Further studies are in progress using the same type of pigs but allowing them to become infected naturally while on one-fifth the therapeutic dose per day.

SUMMARY

One-tenth therapeutic dose per day of polymeric piperazine-1-carbodithioic acid given in the feed one week prior to, and three weeks after, infection failed to prevent migration of *Ascaris lumbricoides* larvae. Respiratory embarrassment and liver scarring resulted, although no worms were recovered from the small intestines of any of the pigs. Uninfected control litter mates showed no liver or lung involvement and their weight gain was slightly greater during the experiment.

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An Emulsion-Type Erysipelas Bacterin
I. Duration of Immunity in Pigs Vaccinated at Weaning

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THERE are several types of immunizing agents available for the protection of swine against infection with *Erysipelothrix rhusiopathiae*. Two of these products, the Lorenze vaccine [1] which is the live virulent culture given simultaneously with immune serum and the Traub adsorbate bacterin [2] which is a formalin-killed, aluminum hydroxide-adsorbed product, are at the present time used extensively in this country. Both products have been proved effective immunizing agents by use in the field and by experimental evaluation.[3, 4]

Each of the two immunizing agents, however, have certain undesirable features. The disadvantage of the Lorenze method, employing a live virulent culture of *Ery. rhusiopathiae* which can infect man as well as several species of animals, is apparent. The Traub adsorbate bacterin has the drawback of conferring an immunity of practical value for only two to four months as demonstrated by laboratory methods.[4] Neither product will give a satisfactory immune response when used in newborn pigs.[5]

The favorable effect on the degree and duration of antibody levels when an antigen is incorporated into an emulsion is well known. The use of light mineral oil as an adjuvant, for this purpose, has been reviewed by Freund.[6] The purpose of this paper is to report the duration of immunity obtained with a formalin-killed, water-in-oil, emulsion-type erysipelas bacterin when inoculated into weanling pigs.

MATERIALS

The adsorbate bacterin used in this experiment was prepared in this laboratory according to methods based on Traub's technique.[2] Immunogenic strains* of *Ery. rhusiopathiae* were used for the production of the bacterin. The individual strains were inoculated into separate flasks containing a horse meat and horse liver infusion medium with peptone, phosphate salts, 5.0 per cent horse serum, and 1.0 per cent glucose at a final pH of 8.0. At the end of 36 hours of growth, 1.5 per cent aluminum hydroxide gel was added in an amount equal to 25.0 per cent of the volume of culture for the purpose of adsorbing and concentrating the antigen. Formalin was then added to make a final concentration of 0.4 per cent. After the aluminum hydroxide-antigen complex had settled, the supernatant, comprising approximately 70.0 per cent of the total volume, was decanted and discarded. The remaining slightly viscous, creamy material was the bacterin which we designated adsorbate bacterin 114.

This bacterin was tested for sterility in thioglycollate liquid medium and was inoculated into mice and pigeons for a safety test. It was found satisfactory in both instances. The immunizing potency was determined on mice by means of the "Potency Test for Erysipelas Bacterin."† The potency test was found to be satisfactory, and the product may be considered as representative and typical of the erysipelas adsorbate bacterins now available in this country.

The emulsion bacterin was prepared from the adsorbate bacterin 114 in the following manner: 370 ml. of adsorbate bacterin 114, 590 ml. of light mineral oil,‡ and 40 ml. of manide monooleate § were placed in an Eppenbach colloid mill and processed for ten minutes to give a stable, water-in-oil emulsion which was designated erysipelas emulsion bacterin 17. The product was found satisfactory in regard to sterility, safety, and potency as determined by the same tests used for the adsorbate bacterin 114.

Mixed-bred weanling pigs, from five different herds, were selected at random for this experiment. These farm herds, located in the Northwest Missouri area, had been previously tested by the scarification technique [7, 8] and found susceptible to swine erysipelas.

The challenge strains of *Ery. rhusiopathiae*, HC585, 8192, 422-1, and NF4,‖ had been satisfactorily used as challenge cultures.[7]

METHOD

The swine were immunized on the farms where they were raised when approximately 8 weeks old.

From Research Laboratories, Inc., Department of Production and Product Development, St. Joseph, Mo.

* Two strains were obtained from Dr. K. Von Bakos, State Veterinary Institute, Stockholm, Sweden, and two were obtained from Dr. R. D. Shuman, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

† United States Department of Agriculture, ARS circular letter ZV, Nov. 18, 1953.

‡ Trade name bayol F, Penola Oil Co., Linden, N. J.

§ Trade name arlacel A, Atlas Powder Co., Wilmington, Del.

‖ Furnished by Dr. R. D. Shuman.

A few pigs in each herd were ear-marked and left unvaccinated for controls. In herds 1 through 4, 5 ml. of erysipelas emulsion bacterin 17 was inoculated intramuscularly into the ham. The 40 pigs in herd 5 were separated into three groups: 15 were given 5 ml. of emulsion bacterin 17 into the ham; 15 were given 5 ml. of adsorbate bacterin 114 into the ham; and 10 were left uninoculated as controls. The immunized and control pigs were left together on the owner's farm and under his care. Balanced rations of home-grown grain and protein supplement were fed.

After immunization, at intervals of 107 to 237 days, the immunity of the principal and control pigs from the different herds was challenged. The animals were routinely brought into the laboratory pens five days prior to challenge and placed on a diet of corn and oats. Their immunity was then challenged, with the method described by Shuman[7] and Fortner and Dinter,[8] by applying the exposure cultures to the scarified, shaved skin, using four vertical scratches, about 12 inches long and 4 inches apart. These scratches were deep enough to allow blood-tinged serous fluid to ooze, but not deep enough to cause frank bleeding. Twenty-four hour broth cultures of either *Ery. rhusiopathiae* strains HC585, 8192, 422-1, or NF4 were rubbed into one of the four scarifications by means of a cotton swab.

The local reaction at each of the four scarification sites on each pig was recorded in the following manner: R for severe (fig. 1A), r for mild, ? for questionable, and O for no reaction (fig. 1B). This scoring was done by one of us without knowledge as to which group the pig belonged. The reaction in a susceptible pig was usually of maximum intensity between 48 to 72 hours; for comparison, the results of the local reaction at 48 hours are included.

Rectal temperatures were also recorded daily to

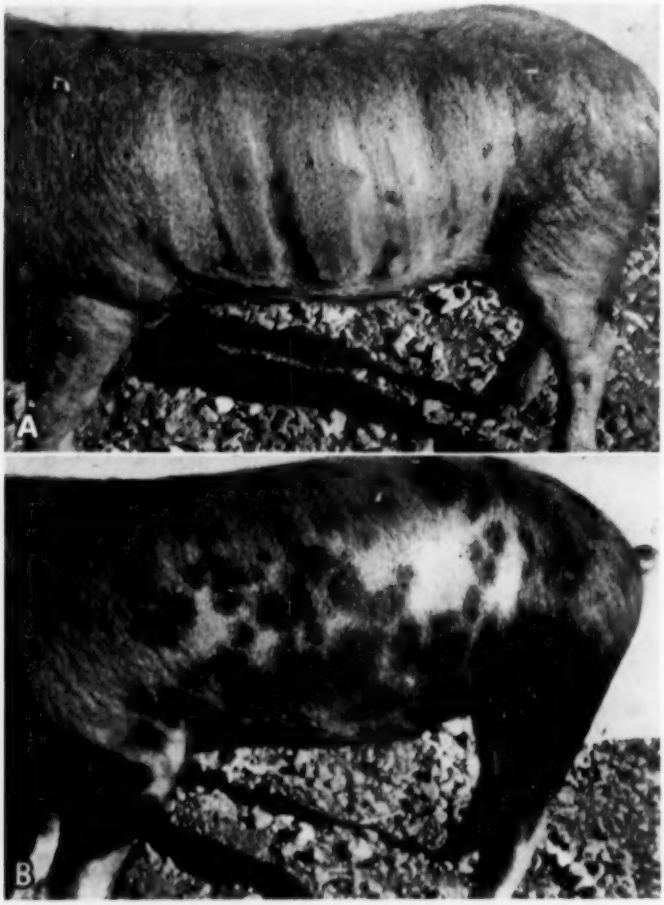

Fig. 1—Results of the scarification method of challenge. (A) Note the severe local reaction (recorded RRRR) at each of the four scarification marks in this erysipelas-susceptible pig (pig 1051, fig. 2). (B) Note the lack of local reaction (recorded OOOO) and the normal healing of the scarification marks in this erysipelas-immune pig (pig 1049, fig. 2).

indicate the generalized reaction, if any. Since the normal temperature for swine in this environment was 102 to 103 F., only those over 104 during the first six days after challenge are shown.

RESULTS

The results of tests on herd 1 are shown alone (fig. 2). The results from herds 2

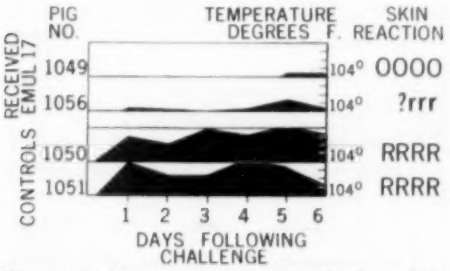

Fig. 2—Resistance of swine from herd 1 challenged 107 days after vaccination with emulsion 17. Weight when challenged averaged 125 lb. Skin reaction scored as follows: R = severe local reaction as seen in figure 1A; r = mild but definite local reaction; ? = slight local inflammation of questionable cause; and 0 = no local infection apparent (fig. 1B).

and 4 are shown combined (fig. 3), since the immunity of both herds was challenged about the same length of time after inoculation. The data from herd 3 were omitted because only 50 per cent of the controls became infected upon challenge. The challenge results on 31 of the original 40 swine in herd 5 are also presented (fig. 4 and 5). The remaining pigs died of gastroenteritis of viral origin shortly after inoculation.

The febrile responses and local reactions of the swine after the challenge were well correlated. They express the comparative degree of resistance to challenge with *Ery. rhusiopathiae*.

Emulsion bacterin 17 elicited a marked degree of protection for at least eight months after vaccination as determined by scarification challenge. The animals immunized with this bacterin which showed a mild local reaction (1056 in fig. 2, 1001 in fig. 4, and 1160 in fig. 5) also showed a slight febrile response, suggesting that a degree of immunity existed.

The pigs vaccinated with adsorbate bacterin 114 were placed in two groups and their immunity was challenged, respectively, 165 and 237 days later. While the immunity from the adsorbate-type bacterin is not expected to last over four months, the swine receiving it did have some resistance, as evidenced by the mild temperatures and local reactions (fig. 4 and 5).

DISCUSSION

The decision to use mixed-bred swine and to hold them on their respective farms was made in an effort to evaluate the efficacy of the vaccine on a common type of swine under field conditions. These mixed-bred swine, however, were found to be less uniform in their susceptibility to erysipelas infection than two purebred herds tested by the authors. Since the pigs in each herd were held under identical environmental conditions and on farms with no previous history of erysipelas infection, it is felt that a natural variation in individual resistance may explain the failure of control pig 1031 (fig. 4) to become infected from the challenge exposure. This would not be unique with this type of host-parasite combination.

The immunizing potency of the emulsion-type bacterin seemed to be superior to that of the adsorbate bacterin. It also appears to be superior to the Lorenze vaccine, which has been reported to have given 80 per cent protection at five months,

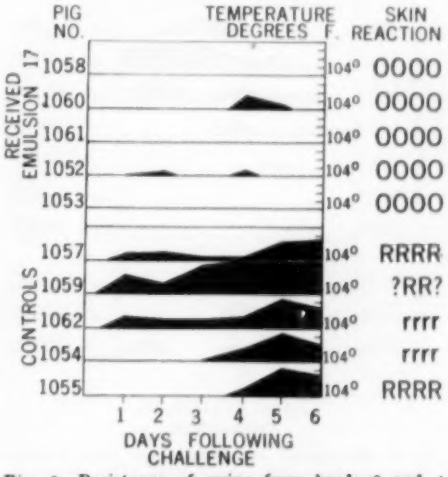

Fig. 3—Resistance of swine from herds 2 and 4 challenged 134 and 136 days, respectively, after vaccination with emulsion 17. Pigs 1058, 1060, 1061, 1057, 1059, and 1062 from herd 2 averaged 186 lb. when challenged. The remaining pigs, herd 4, averaged 175 lb. (See fig. 2 for key to local reactions.)

20 per cent at six months, and again 80 per cent in the group tested at seven months.[9]

This superior potency of the emulsion-type bacterin is due to the incorporation of the antigen into an emulsion and is, to a certain degree, independent of the amount of antigen present. Emulsion bacterin 17 which was used in these experiments had only 37 per cent as much antigen present as a corresponding 5-ml. dose of adsorbate bacterin 114. Subsequent unpublished data indicate that the amount of antigen can be reduced still further (at least 40% further) without affecting the immunizing potency.

One additional aspect of the results warrants mention. The swine vaccinated with adsorbate bacterin 114 appeared to

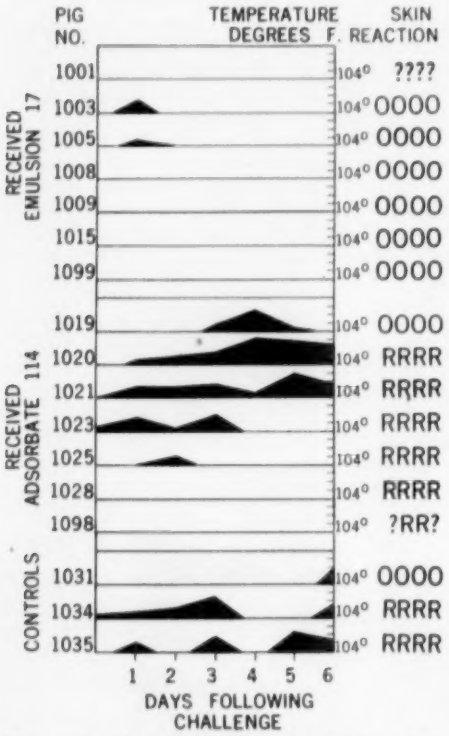

Fig. 4—Resistance of swine from herd 5 challenged 165 days after vaccination. One group received emulsion 17 and one group received adsorbate 114. Weight when challenged averaged 232 lb. (See fig. 2 for key to local reactions.) The small number of controls was the result of disproportionate losses due to gastroenteritis several weeks after vaccination.

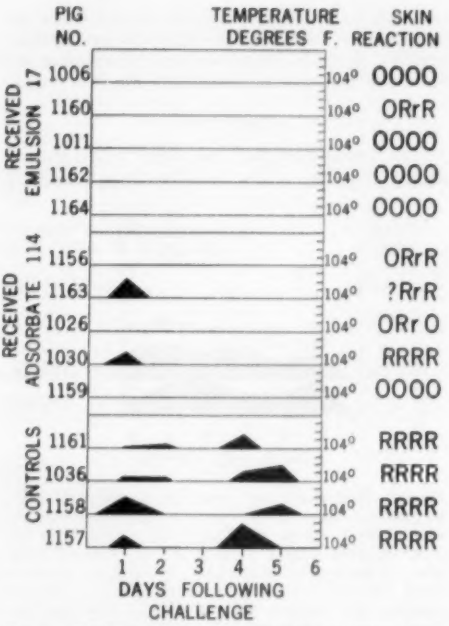

Fig. 5—Resistance of the remainder of swine from herd 5 challenged 237 days after vaccination. One group received emulsion 17 and one group received adsorbate 114. Weight when challenged averaged 360 lb. (See fig. 2 for key to local reactions.)

have more resistance at 237 days (fig. 5) than at 165 days (fig. 4). The swine receiving emulsion bacterin 17 (fig. 2 to 5) also showed progressively less response to exposure as the time between vaccination and challenge lengthened. In the four groups, during the period of 107 to 237 days after vaccination, the percentage of swine showing a fever was, respectively, 100, 40, 29, and 0. It appeared as though both bacterins produced a greater immunity as the interval before challenge increased. This anomaly might be explained by considering the difference in the response to challenge exposure of swine of different sizes. This febrile response is less severe in the larger swine although the local skin reaction is approximately the same for swine of all sizes (control pigs in fig. 2 to 5). In addition to the febrile aspect, it was observed while exposing several hundred control pigs, that it is not unusual for pigs weighing less than 100 lb. to develop a clinical case of chronic erysipelas if left untreated fol-

lowing challenge exposure. This was never observed in larger swine. The combined effect of the vaccines plus the increasing resistance of older swine to this type of challenge exposure caused the vaccine-induced immunity to appear greater as the time following vaccination increased.

(The significance of the local reaction and the hypersensitivity resulting from an inoculation with a water-in-oil emulsion vaccine are discussed in a companion paper.[10])

SUMMARY

A formalin-killed, aluminum hydroxide-adsorbed, *Erysipelothrix rhusiopathiae* bacterin has been combined with the emulsifying agent, manide mono-oleate (arlacel A), and a light mineral oil (bayol F) to form an oil-in-water, emulsion-type bacterin. This emulsification process has enhanced the immunizing potency of the *Ery. rhusiopathiae* antigen considerably. Mixed-bred pigs inoculated at weaning age with the emulsion bacterin developed a practical immunity lasting at least 237 days. This immunity was shown to be superior to that produced by the adsorbate bacterin and it is superior to that reported for the Lorenze (live virulent culture and serum) vaccine.[9]

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An Emulsion-Type Erysipelas Bacterin. II. Duration of Immunity Following Vaccination of Newborn Pigs

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THE FACT that newborn pigs are susceptible to infection with *Erysipelothrix rhusiopathiae* has been reported many times.[1-4] The practical value of vaccinating newborn pigs against erysipelas with anti-erysipelas serum and live virulent erysipelas culture has been discussed by Shuman.[5] He has shown, by using the scarification method of immunity challenge,[5,6] that pigs vaccinated when 2 to 5 days old have a limited immunity at weaning time and no significant immunity at market age.[6] With weanling pigs, the erysipelas adsorbate bacterin has been shown, by laboratory methods, to confer an immunity lasting two to four months,[9] compared to an immunity duration of six to seven months when vaccinated with the live virulent erysipelas culture and serum.[10] The live avirulent erysipelas vaccines have been found in Europe to have approximately the same degree of potency as the adsorbate vaccine.[11-13] Hence, we could not expect better results in newborn pigs with the avirulent or adsorbate immunizing agents than Shuman reported with the virulent live culture and immune serum method. There is one report, unconfirmed by others, on a superior avirulent live culture erysipelas vaccine produced in this country.[14]

It appears that there is no erysipelas-immunization procedure requiring only a single treatment, which can be expected to give immediate protection to newborn pigs, as well as a durable immunity lasting until market time. The purpose of this work is to investigate one approach to this problem.

It has been demonstrated that when the erysipelas adsorbate bacterin is incorporated into a water-in-oil emulsion, it becomes a better immunizing agent.[15] This emulsion bacterin gave a strong immunity of practical value for at least eight months after the vaccination of weanling pigs. This paper presents the results of using the emulsion-type erysipelas bacterin, with and without anti-erysipelas serum, on newborn pigs.

MATERIALS

The erysipelas adsorbate bacterins used in these experiments, and designated as adsorbate bacterin 114 and adsorbate bacterin 15, were prepared in an identical manner. Emulsion bacterin 17 was prepared using adsorbate bacterin 114 as the aqueous phase, while for emulsion bacterin 41 adsorbate bacterin 15 was used as the aqueous phase. (Detailed information on the production and testing is given in the preceding paper.[15])

The anti-erysipelas serum used was prepared in this laboratory in horses, according to accepted commercial technique. The serum was demonstrated to be sterile, and it satisfactorily passed the potency test on pigeons.*

The *Ery. rhusiopathiae* live virulent culture 1 was supplied by a leading reputable commercial producer of biological products.

The first 42 pigs used, in tests 1 and 2 (fig. 1 and 2), were from 6 crossbred gilts, selected from various local herds, and a Hampshire boar. They were farrowed within five days in August, 1954.

The same 6 sows, along with 2 additional erysipelas-susceptible sows, were bred again to the same boar six weeks after farrowing. In January, 1955, the 8 sows farrowed 73 pigs which were used for tests 3, 4, and 5 (fig. 3, 4, and 5).

The challenge strains of *Ery. rhusiopathiae* were the same as used before.[15]

METHOD

The litters of the August, 1954, farrowing were held with their respective dams in separate pens. The pigs were identified by ear notches and were assigned to three groups (a, b, and c) consisting of approximately equal numbers of pigs from each litter. These pigs were vaccinated four days after the last sow farrowed, when all were between 4 and 9 days old.

Group a.—These 18 pigs were given 5.0 ml. of emulsion bacterin 17, divided equally into each axillary space.

Group b.—These 12 pigs were given 5.0 ml. of adsorbate bacterin 114 in the same manner.

Group c.—This group of 12 pigs was left uninoculated to serve as controls.

When the pigs were 8 weeks old, they were weaned and vaccinated for hog cholera. They were

From Research Laboratories, Inc., Department of Production and Product Development, St. Joseph, Mo.

* As set forth in the Virus-Serum Control Memo 50-5 ZV, June 1, 1950, BAI (now the ARS), U. S. Department of Agriculture, Washington, D. C.

then put together on pasture and fed home-grown grain with a protein supplement.

Test 1.—At 111 days after vaccination, the immunity of approximately one half of the pigs from groups a, b, and c was challenged (fig. 1).

Test 2.—At 193 days after vaccination, the immunity of the remaining pigs of groups a, b, and c was challenged (fig. 2).

The 73 pigs of the January, 1955, farrowing were likewise identified by ear notches and were assigned to five groups (d, e, f, g, and h), each group containing approximately equal numbers of pigs from each litter. The pigs were vaccinated when 7 to 14 days old, since the youngest were 7 days old and the sows had farrowed over a seven-day period.

Group d.—The 14 pigs in this group were each given 5.0 ml. of emulsion bacterin 41 divided equally into each axillary space.

Group e.—These 13 pigs were likewise given 5.0 ml. of emulsion bacterin 41, simultaneously with 5.0 ml. of anti-erysipelas serum given intraperitoneally.

Group f.—These 14 pigs were given 5.0 ml. of adsorbate bacterin 15 in divided doses.

Group g.—These 12 pigs were given 0.5 ml. of commercial live virulent culture 1 into one axillary space simultaneously with 3.0 ml. of anti-erysipelas serum given intraperitoneally.

Group h.—This group of 20 pigs was left uninoculated to serve as controls.

These pigs were cared for like the previous group and were vaccinated for hog cholera at 8 weeks of age.

Test 3.—At 247 days after vaccination, the immunity of 4 pigs from group d and 3 control pigs from group h was challenged (fig. 3). The remaining pigs of group d were not used in this experiment.

Test 4.—At 182 days after vaccination, the immunity of some of the pigs in groups e, f, g, and h was challenged (fig. 4).

Test 5.—At 247 days after vaccination, the immunity of the remaining pigs from groups e, f, g, and h was challenged (fig. 5).

During the periods between vaccination and challenge, 4 pigs were withdrawn, 2 each from groups a and e, for slaughter to study the local response to the immunizing agent, and 2 pigs died. The clinical and laboratory diagnosis on the 1 which died at 5 weeks of age was salmonellosis; the second pig was not in suitable condition for a diagnosis. The remaining pigs were in good health during this period.

Five days prior to challenge, the animals were brought in from the Research Laboratory Experimental Farm to the laboratory pens and placed on a ration of corn and oats. Their immunity was then challenged by the skin scarification method.[6, 7, 16]

Their local and generalized reactions were recorded (fig. 1 to 5). (The nature of the response to the challenge, as well as the method of recording this response, is discussed in the preceding paper.[15])

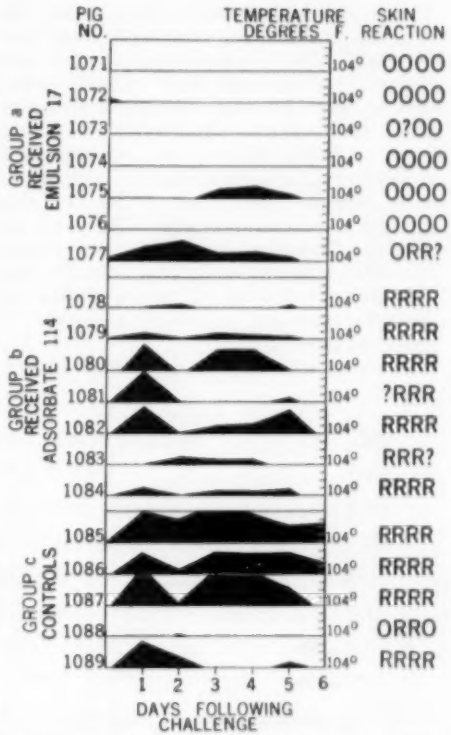

Fig. 1—Results of test 1: Resistance of swine to erysipelas infection 111 days after vaccination (without the simultaneous use of anti-erysipelas serum). Weight of pigs at challenge averaged 85 lb. The local reaction occurring at each of the four scarification marks were scored as follows: R = a severe local reaction; r = a mild local reaction; ? = a slight local inflammation of questionable cause; and O = no local infection apparent.

RESULTS

Vaccination Without Simultaneous Use of Anti-Erysipelas Serum.—The vaccinated animals of tests 1, 2, and 3 (fig. 1 to 3) did not receive immune serum simultaneously with the bacterin. In tests 1 and 2, group b was included to assess the activating effect of incorporating this same adsorbate bacterin into a water-in-oil emulsion. When it became obvious that the adsorbate bacterin was not giving a durable immunity, this group was omitted from test 3.

Figures 1 to 3 indicate clearly that the water-in-oil, emulsion-type erysipelas bacterin will give a durable practical immunity lasting at least eight months when used on newborn pigs. While several pigs which had received emulsion bacterin did show evidence, after challenge exposure, of mild erysipelas infection, only 1 pig of these groups (1077, test 1, fig. 1) succumbed to severe erysipelas. The mild 48-hour skin reaction on emulsion-vaccinated pigs 1115 (fig. 2) and 1208 (fig. 3) was completely faded at 72 hours, indicating that the animals possessed a degree of practical immunity.

The pigs in tests 1 and 2 which had received the adsorbate bacterin 114 did show a slightly less severe febrile reaction than the controls, but the difference was of little significance.

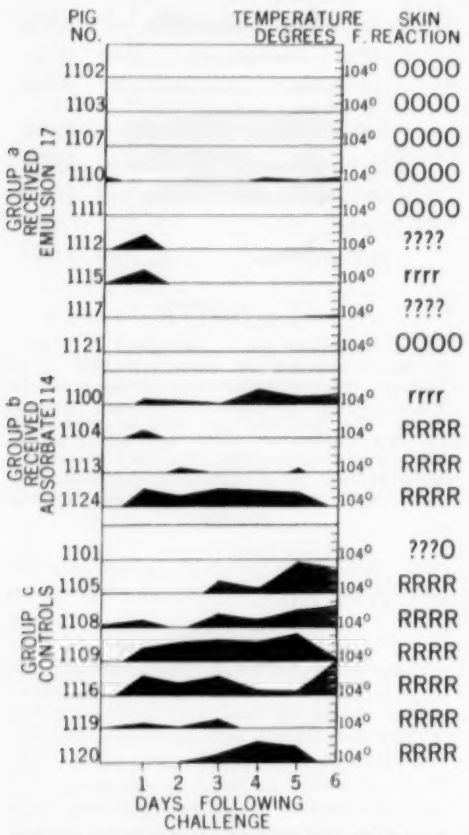

Fig. 2—Results of test 2: Resistance of swine to infection 193 days after vaccination (without the simultaneous use of anti-erysipelas serum). Weight of pigs at challenge averaged 179 lb. (See fig. 1 for key to local reactions.)

These results demonstrate, as did the earlier work,[15] that the febrile response in the control pigs, as well as in the various vaccinated pigs, decreases as the pigs become older. No increase in resistance, with age, to the local reaction has been observed. While this may suggest a greater resistance to erysipelas as the pig ages, it is not in accord with the generally prevailing opinions.[17]

Vaccination with the Simultaneous Use of Anti-Erysipelas Serum.—Tests 4 and 5 (fig. 4 and 5) were conducted to determine the practicability of using immune serum simultaneously with the emulsion bacterin. The pigs in group f (adsorbate bacterin 15 and serum) and group g (live virulent culture 1 and serum) were included for comparison. The pigs in groups e and f received 5.0 ml. of serum. The pigs of group g were vaccinated with 0.5 ml. of live virulent culture and 3.0 ml. of serum. This dosage relationship between the culture and serum was shown by Shuman[10] to give excellent results when used on weanling pigs.

The results (fig. 4 and 5) indicate that the emulsion bacterin may be given simultaneously with anti-erysipelas serum and still produce an immunity lasting over eight months. As indicated in tests 1 and 2, the adsorbate bacterin with serum did not give a durable immunity. The pigs vaccinated with live virulent culture and serum, as shown by Shuman,[8] did not develop a practical, durable immunity.

In test 4, 1 emulsion-vaccinated pig (1174, fig. 4), of the group challenged at 182 days after vaccination, did show a mild skin reaction at 48 hours which faded completely by 72 hours. However, 1 pig (1222, fig. 5) in the emulsion-vaccinated

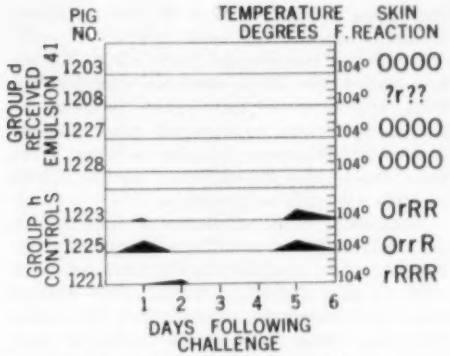

Fig. 3—Results of test 3: Resistance of swine to infection 247 days after vaccination (without the simultaneous use of anti-erysipelas serum). Weight of pigs at challenge averaged 276 lb. (See fig. 1 for key to local reactions.)

group was susceptible when challenged 247 days after vaccination.

In both tests 4 and 5 (fig. 4 and 5), the pigs which did not receive the emulsion bacterin (groups f, g, and h) demonstrated complete susceptibility.

DISCUSSION

The potentiating effect of incorporating an antigen into a water-in-oil emulsion is well established, as indicated in the review by Freund.[18] The reason for enhancement of the immunity response is not completely understood. The oil phase acts as a protective coating around the antigen, thus delaying its being phagocytized and eliminated[19,20] and resulting in a prolonged antigenic stimulation. The degree and importance of any inflammatory process elicited by the oil adjuvant has not been determined.

Unfortunately, one property of water-in-oil emulsion vaccines has presented a problem which has somewhat discouraged their use in man; they have, on occasion, caused a persistent localized nodule at the point of inoculation. Another disturbing aspect of such emulsion preparations is their striking effect on sensitization.

The appearance of nodules following inoculations with emulsion immunizing products has been reported several times.[21-23] These nodules, which were frequently purulent, occurred in many of the patients and often persisted for many months. The emulsions used in these studies were made with various mineral oils and falba.† Later reports by Salk and his group[24-27] indicated that by using a finer grade of oil (bayol F) and purified manide mono-oleate (arlacel A) and by administering the inoculation intramuscularly the untoward local reaction may be eliminated.

† Distributed by Pfaltz and Bauer, Inc., New York, N. Y.

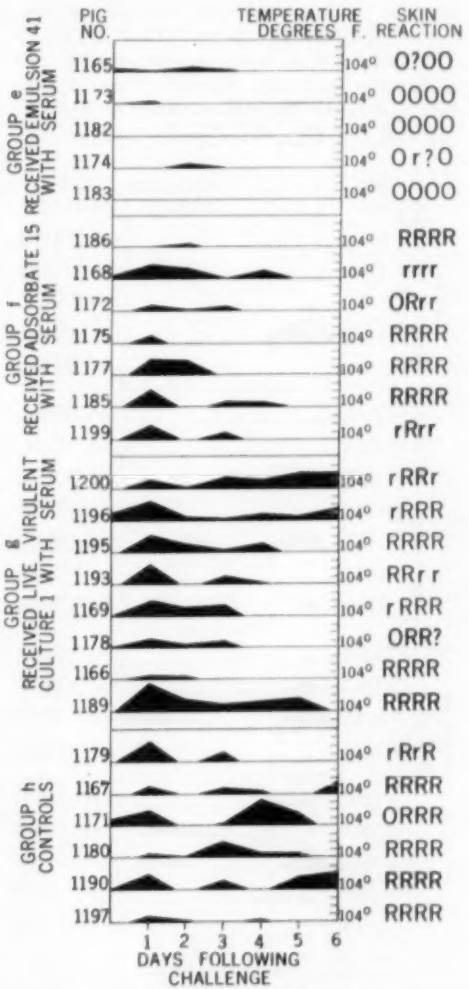

Fig. 4—Results of test 4: Resistance of swine to infection 182 days after vaccination (with the simultaneous use of anti-erysipelas serum). Weight of pigs at challenge averaged 198 lb. (See fig. 1 for key to local reactions.)

In this experiment, the emulsion bacterin was inoculated into the axillary space. Over 30 of these animals were slaughtered at various intervals after inoculation and the product was observed to cause a purulent (sterile), noninflammatory abscess in approximately 60 per cent of the pigs. The purulent material varied from about 1 to 3 cc. The abscesses gradually decreased in size but were observed for as long as four months. Subsequent work in rabbits confirmed the earlier reports [22] that the bayol F and arlacel A were not responsible *per se* for the reaction. Further tests on rabbits indicated that the aluminum hydroxide and the *Ery. rhusiopathiae* organisms, individually and together, in the emulsion, would cause the reaction.

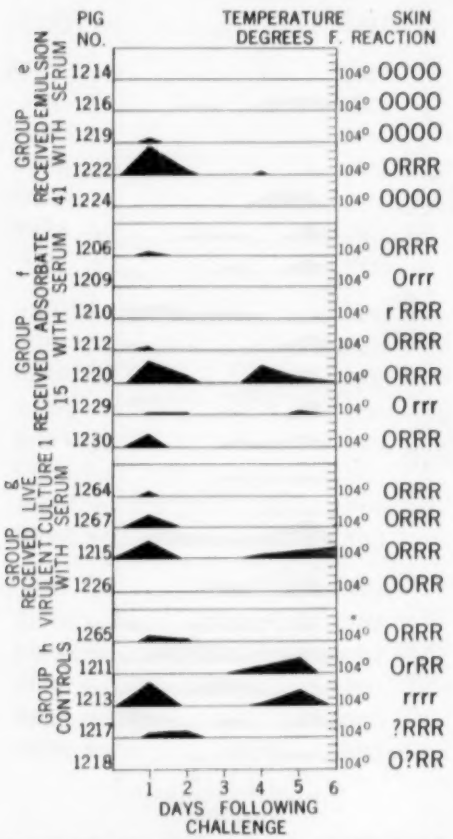

Fig. 5—Results of test 5: Resistance of swine to infection 247 days after vaccination (with the simultaneous use of anti-erysipelas serum). Weight of pigs at challenge averaged 276 lb. (See fig. 1 for key to local reactions.)

Reducing the dosage from 5.0 to 2.0 ml. minimized the local reaction measurably. Pigs appeared to have just as good an immunity when challenged four months later with this reduced dose as when given the 5.0-ml. dose of emulsion vaccine. These studies are still in progress.

Salk *et al.*[24,25] demonstrated that minute amounts of antigen will elicit antibody formation when combined with oil in an emulsion. Using influenza virus, they showed that approximately one to ten thousandfold less virus, beyond the point of antigenic extinction of virus in saline, were still active in an emulsion. The 2.0-ml. dose of erysipelas emulsion bacterin, which seemed to stimulate a durable immunity, has about 14 per cent as much antigen as the standard 5.0-ml. dose of aqueous adsorbate bacterin. It would appear possible to reduce the erysipelas antigenic material to a point where all local irritation would be eliminated and yet preserve the superior immunizing qualities of the bacterin. Further studies should clarify this point.

It has been recognized for years that when an antigen is combined with certain adjuvants, it is capable, upon injection, of producing sensitization. Specifically, it has been shown that the addition of killed mycobacteria to antigens emulsified in mineral oil increases greatly the sensitizing power of the antigen.[19] It is of interest that the emulsion itself is not inclined to cause the antigen to sensitize except when the mycobacteria are present.[18] It also has been stated that equine serum emulsified with paraffin oil and falba will have little, if any, effect on sensitization.[26] This is interesting since, quite by accident, we noticed that pigs vaccinated with our erysipelas emulsion bacterin became sensitized to equine serum. The seriousness and potential danger of this type of situation is obvious when one realizes the importance and extent to which serum therapy is used in veterinary medicine.

The aqueous phase of the erysipelas emulsion bacterin used in these experiments contained the following material: horse meat and horse liver infusion, aluminum hydroxide, peptone, equine serum,‡

‡ Each 5.0-ml. dose of erysipelas emulsion bacterin contained approximately 0.25 ml. of equine serum originating from the mediums used to grow the *Ery. rhusiopathiae*.

gelatin, and *Ery. rhusiopathiae* cells and soluble antigen from those cells. Pigs immunized with 5.0 ml. of the emulsion bacterin became sensitized to the extent that as little as 5.0 ml. of equine serum given intramuscularly into the ham four months later could kill 250-lb. hogs—many of them within five minutes. They died with typical anaphylactic reaction, in spite of adrenalin and antihistamin therapy. In addition to this, it was demonstrated that bovine serum and purified bovine albumin[§] would also produce shock in these pigs. Conversely, emulsions in which bovine serum had been used in the original culture mediums in place of equine serum would sensitize the animal to equine serum, as well as bovine serum. It would be premature to attempt to draw conclusions concerning specificity until the results can be duplicated, using a single source of protein for sensitization. These observations emphasize the necessity for caution in marketing emulsion-type vaccines and bacterins.

The role of aluminum hydroxide and *Ery. rhusiopathiae* in this phenomenon is under investigation. Considering the phylogenetic relationship between *Ery. rhusiopathiae* and the Mycobacterium, one is inclined to suggest that they are similar in their capacity to induce sensitization in an animal when incorporated with other antigenic material into an emulsion.

By the elimination of certain constituents in the erysipelas emulsion bacterin, or by using homologous serum in the medium used for growing the erysipelas organisms, it is anticipated that a vaccine can be produced which will not cause serum sensitization.

SUMMARY

Pigs vaccinated when 4 to 14 days old with this erysipelas emulsion bacterin developed an immunity lasting for at least eight months. The duration of the immunity was not affected when anti-erysipelas serum was given simultaneously with the emulsion-type bacterin. Both virulent erysipelas culture and serum and erysipelas adsorbate bacterin failed to produce a practical immunity under the same conditions.

There were two undesirable features of the emulsion bacterin: (1) the tendency to cause persistent local reaction and (2) the ability to cause hypersensitivity, in the pig, to the proteins in the bacterin. The possible methods of overcoming these objectional features are discussed.

§ Bovine plasma *delta* albumin 5 per cent solution, Armour Veterinary Laboratories, Kankakee, Ill.

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Strain Variation in the Response of Sheep Nematodes to the Action of Phenothiazine. I. Studies of Mixed Infections in Experimental Animals

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THE EARLY STUDIES on phenothiazine as an anthelmintic in sheep[7,10,13,18,25] established the efficacy of therapeutic doses in removing nematodes from the gastrointestinal tract. Subsequent observations, by Shorb and Haberman,[22] indicated the effectiveness of small daily doses of phenothiazine in inhibiting the development of roundworm larvae in the feces, which led to the use of salt mixtures[11] as a feasible means of administering the drug. From these and other studies,[6,12,26] the general pattern of action of small daily doses of phenothiazine became apparent and the principal effects of suppressing egg production and inhibiting larval development were pointed up. There was also some evidence of a slow, killing effect on the adult worms and an adverse activity against ingested larvae. Numerous investigations[6,12] attest to the value of phenothiazine for the control of the gastrointestinal nematodes of sheep, and various schedules or combinations of drenches and salt or mineral mixtures have been widely used.

Concern has increased[2,3,12] over the development of drug-fast strains of nematodes from the prolonged and intensive use of phenothiazine, but there has been no evidence of this. Sinclair[23] was unable to demonstrate resistance to therapeutic doses of phenothiazine in *Trichostrongylus colubriformis* of sheep in New Zealand. The continued effectiveness of phenothiazine-salt mixtures over extended periods has been observed.[16,17,27]

In 1953, field reports[20] from the central Kentucky region indicated that phenothiazine was no longer effectively controlling the parasites of sheep. Field observations on representative flocks in this area, in 1954,[4] revealed that the highest level of worm infection, as indicated by bimonthly fecal egg counts, was in the flock in which phenothiazine was used most extensively. Accordingly, the present study, under controlled laboratory conditions, was undertaken as a follow-up investigation of these field observations.

MATERIALS AND METHODS

Experiments were designed to compare a strain of nematodes (strain A) which had a minimum exposure to phenothiazine with the above-mentioned strain of worms designated strain B. Strain A was from departmental ewes, which had not been medicated with phenothiazine during the preceding three years. In the flock from which strain B was obtained, phenothiazine had been relied on, both in salt mixtures and in periodic drenches, as the principal nematode control procedure for the preceding ten-year period.

Eight lambs (Cheviot crossed with grade western) of mixed sexes were used. They had been raised helminth-free by removal from the ewes at birth and continual confinement in elevated wire-bottom cages. In each of two experiments, 2 lambs were infected with each strain of worms and 1 of each pair was untreated to serve as a control. The lambs were 3 to 5 months old when experimentally infected and their weights were from 39 to 55 lb. in experiment 1; 50 to 71 lb. in experiment 2.

Experiment 1 lambs were infected with 10,000 larvae cultured from feces that were collected directly from the rectums of several of the ewes in the respective flocks in the spring of 1954. Experiment 2 lambs were infected at a later date with approximately 10,000 larvae cultured from the control lambs of experiment 1.

Light infections with *Strongyloides papillosus* of both strains were established in lambs in experiment 1, and lighter infections in experiment 2. The numbers of eggs and larvae of this species were recorded separately and are not included in the total egg and larval counts in the following discussion.

The same lot of a commercial preparation of phenothiazine (N.F., green) was used throughout this study. The small doses were weighed out in 000 gelatin capsules and given daily with a balling gun. The therapeutic doses were suspended in water and administered with a Whitlock, nozzle-equipped, dose syringe. Routinely, the drug administration and fecal sampling were at 4:00 p.m.

This investigation is connected with a project of the Kentucky Agricultural Experiment Station, Lexington, and is published with the permission of the director of the Station and the chief of the Animal Disease and Parasite Research Branch, ARS, U. S. Department of Agriculture, Washington, D. C.

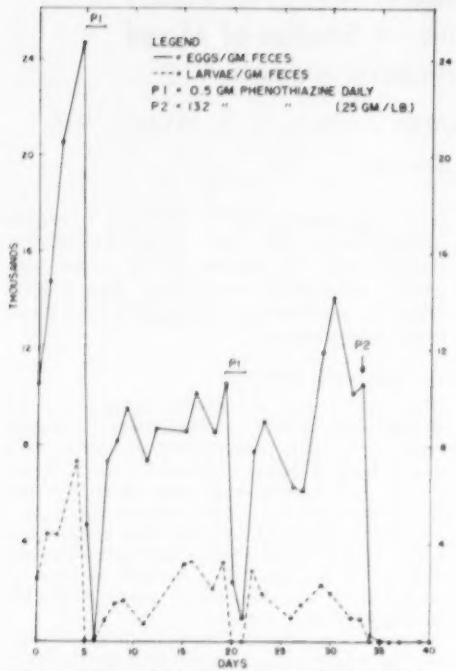

Fig. 1—Egg and larval counts on lamb 48A (experiment 1) infected with strain A mixed nematodes.

Included in the observations on the lambs were daily worm egg and larval counts on the feces, before, during, and after medication, and finally a necropsy to determine the number and species of worms remaining in the gastrointestinal tract. Sufficient feces were taken each day to provide a 5.0-Gm. sample for the flotation egg count and a 5.0-Gm. sample for a larval culture. For the latter, mixtures with charcoal (experiment 1) and sphagnum moss (experiment 2) were prepared in 4-oz., screw-capped jars, and the cultures were routinely put through a Baermann apparatus at seven days for the estimation of the total number of infective (third-stage) larvae by dilution counts.

Egg counts were made by modifying Stoll's salt-flotation method[24] to provide dilution factors ranging from 10 to 50. Differential egg counts[14, 21] were made but only the total counts are presented in this report. In experiment 1, *Haemonchus contortus* eggs accounted for an average of 99.1 per cent of the eggs in lambs given strain A and 88.4 per cent in the lambs given strain B; in experiment 2, for over 99.0 per cent of the eggs in lambs given both strains. The increase, in experiment 2, in the proportion of *H. contortus* in strain B, was apparently due to the failure of Ostertagia and Chabertia to become established in those 2 lambs.

The number of nematodes found at necropsy was estimated by a careful search through aliquot portions of the contents and rinses of the gastrointestinal tract for the smaller forms (Ostertagia, Trichostrongylus, and Cooperia) and a complete examination of the material for the larger forms (Haemonchus, Chabertia, and Oesophagostomum). To facilitate the search for *Trichostrongylus axei*, the abomasal wall was digested with artificial gastric juice, and aliquots of the digest were examined microscopically (x 13).

The bases for comparing the response of the two strains to the action of the drug were (1) depression of egg counts and larval development during treatment; (2) recovery of these after treatment; and (3) number of nematodes recovered at necropsy.

RESULTS

Experiment 1.—The egg (e.p.g.) and larval (l.p.g.) counts on the 4 lambs in this experiment are presented (fig. 1 to 4).

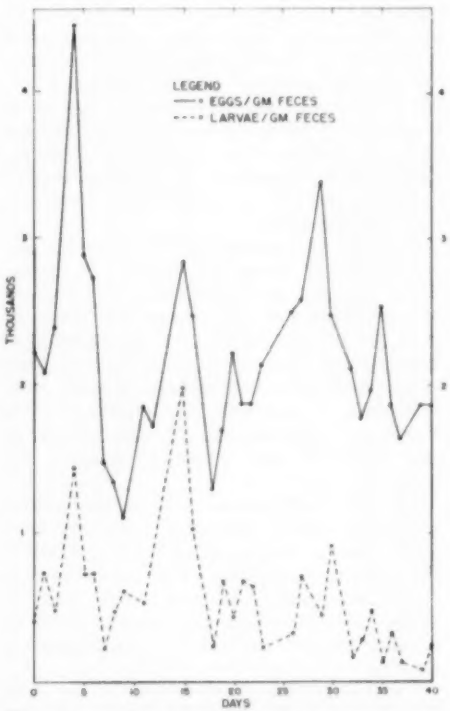

Fig. 2—Egg and larval counts on lamb 48B (experiment 1) infected with strain A mixed nematodes.

With few exceptions, counts were made daily over the 40-day period and the observations were started on the twenty-seventh day after infection.

A striking reduction (86.0%) in e.p.g. was associated with the first course of 0.5-Gm. daily doses of phenothiazine in strain A-infected lamb 48A (fig. 1). Repeating this treatment after a two-week interval produced a similar drop (81.0%) in e.p.g. Following both courses of treatment, recovery in e.p.g. was recorded at 48 hours after the second dose of the drug. Complete inhibition of larval development was observed during both courses of treatment, which was followed by a recovery corresponding with the post-treatment return of the e.p.g.

A therapeutic dose (0.25 Gm./lb.) of phenothiazine to lamb 48A resulted in a nearly complete elimination of eggs and larvae from the feces at 24 hours, which continued until the close of the experiment, one week after dosing.

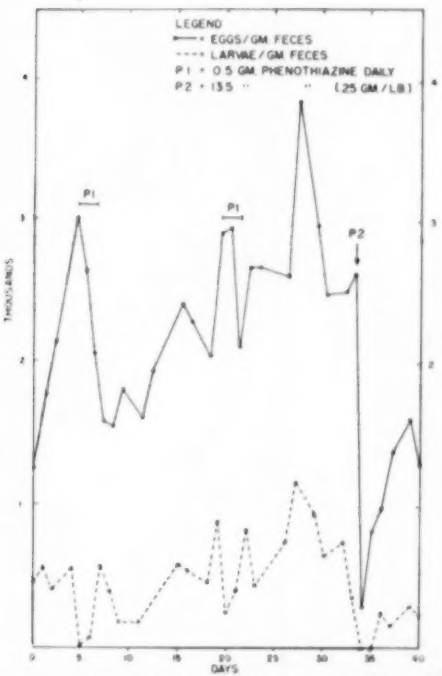

Fig. 3—Egg and larval counts on lamb 410A (experiment 1) infected with strain B mixed nematodes.

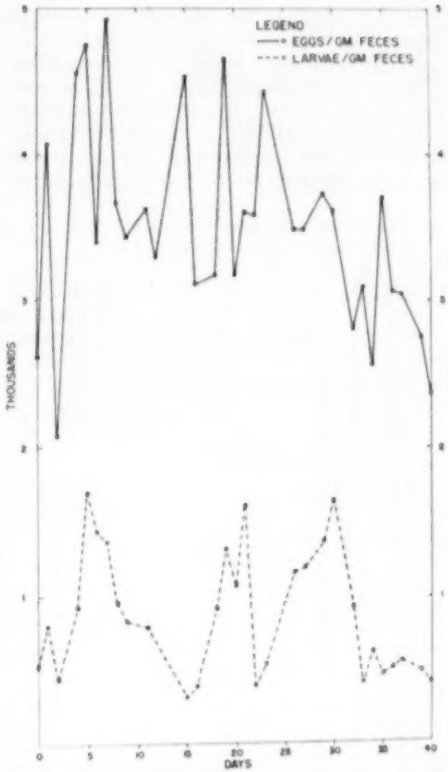

Fig. 4—Egg and larval counts on lamb 410B (experiment 1) infected with strain B mixed nematodes.

There was no appreciable change in e.p.g. on strain B-infected lamb 410A (fig. 3) during the two courses of 0.5-Gm. daily dose treatments. The average of the e.p.g. during the two-day medication periods was 14.8 and 15.3 per cent higher than the respective pretreatment average e.p.g. Inhibition of larval development was practically complete (93.0%) during the first treatment period, but considerably less inhibition (47.0%) was evident during the second course of treatment.

A therapeutic dose (0.25 Gm./lb.) in lamb 410A resulted in a marked reduction in e.p.g. and complete inhibition of larval development at 24 hours. However, the e.p.g. and l.p.g. gradually increased during the ensuing week and tended to level off at about one half the predosing level.

The egg and larval counts on the un-

treated control lambs for strains A and B are shown (fig. 2 and 4, respectively).

All 4 lambs were killed seven days after therapeutic dosing of 48A and 410A and the worms in the gastrointestinal tracts were recorded (table 1). *Haemonchus contortus* was the predominant species found in both control lambs but lambs given strain B had a larger variety of species. The worm collections from the treated animals were generally in accord with the post-treatment changes in the egg and larval counts, which indicated a nearly complete elimination of worms from lamb 48A (strain A) but only a partial removal from lamb 410A (strain B). There was no evi-

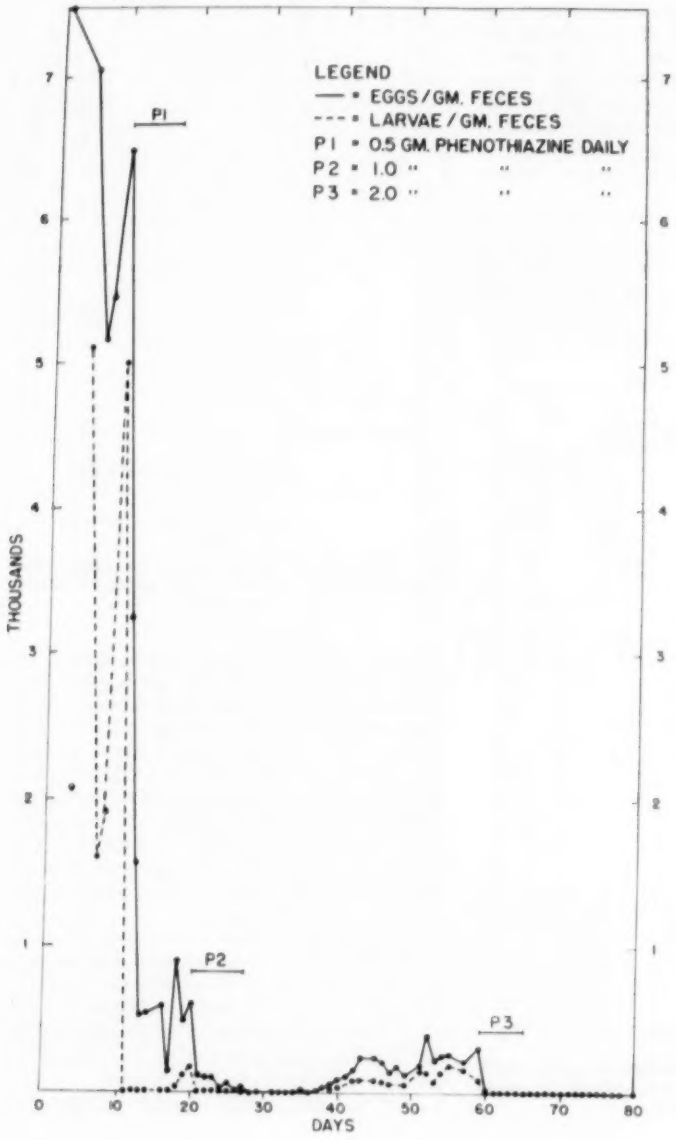

Fig. 5—Egg and larval counts on lamb 4H (experiment 2) infected with strain A mixed nematodes.

dence of species-selective action by the drug in the latter lamb.

The female *H. contortus* were classified according to their vulval process types, as outlined by Roberts *et al.*[19] Of the 60 females from control lamb 48B (strain A), 82.0 per cent were sheep strain, types A, B, or C, and 18.0 per cent were cattle strain, type F, and of the 123 females from the strain B control lamb (410B), 67.0 per cent were types A, B, or C and 33.0 per cent were type F. In close agreement, of the 67 females from the strain B-treated lamb (410A), 66.0 per cent were types A, B, or C and 34.0 per cent were type F.

Experiment 2.—For this experiment, the

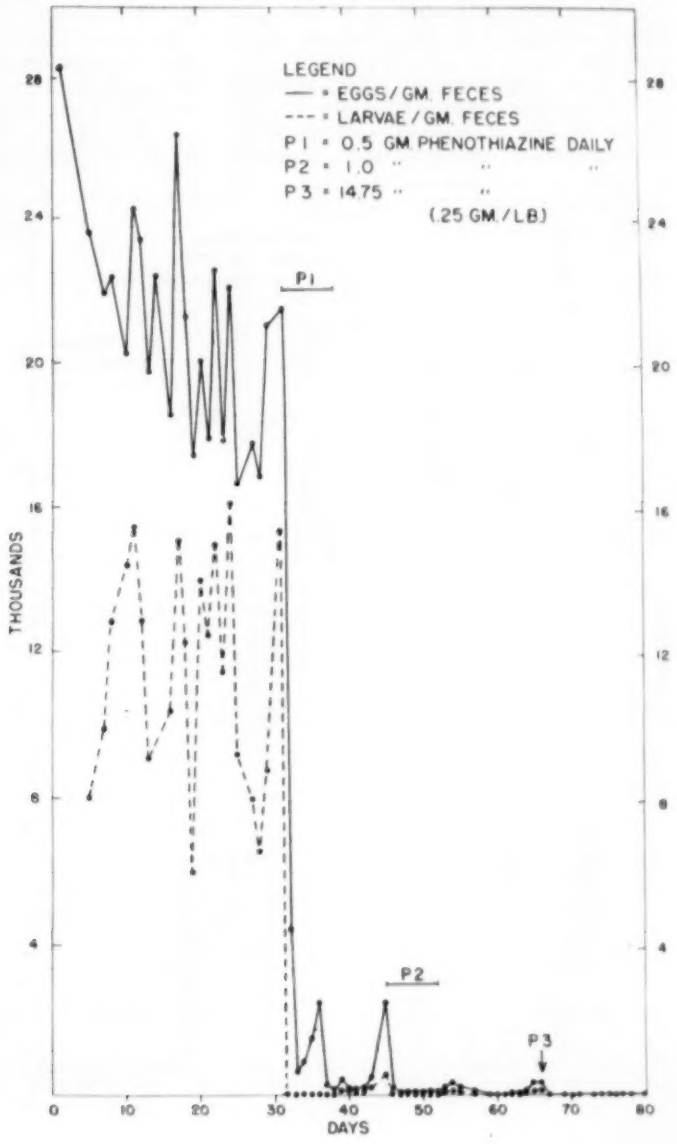

Fig. 6—Egg and larval counts on lamb 4W (experiment 2) infected with strain A mixed nematodes.

courses of small daily doses of phenothiazine were lengthened to seven days and both animals of each pair were treated at intervals, alternately serving as controls. The e.p.g. and l.p.g. observations on these lambs are presented (fig. 5 to 8). For the most part, daily egg and larval counts on the 4 lambs were made over the three-month period covered by the experiment, and preliminary counts were started on the forty-fifth day after infection with larvae cultured from the controls of experiment 1.

Striking reductions in e.p.g. were observed in strain A-infected lambs 4H (fig. 5) and 4W (fig. 2) when given the 0.5-Gm. daily course of phenothiazine. These reductions averaged 87.0 per cent with little recovery of e.p.g. during the post-treatment periods. Increasing the dose level to 1.0 Gm. was associated with further reductions of the egg counts during and after treatment. Subsequently, the eggs were nearly completely eliminated from the feces of these lambs by the 2.0-Gm. daily dose course of therapy of lamb 4H and by a therapeutic dose (0.25 Gm./lb.) to lamb 4W.

Complete inhibition of larval development was also characteristic of the cultures prepared during each of the various treatments of strain A-infected lambs 4H (fig. 5) and 4W (fig. 6). The return of low-grade larval development during the post-treat-

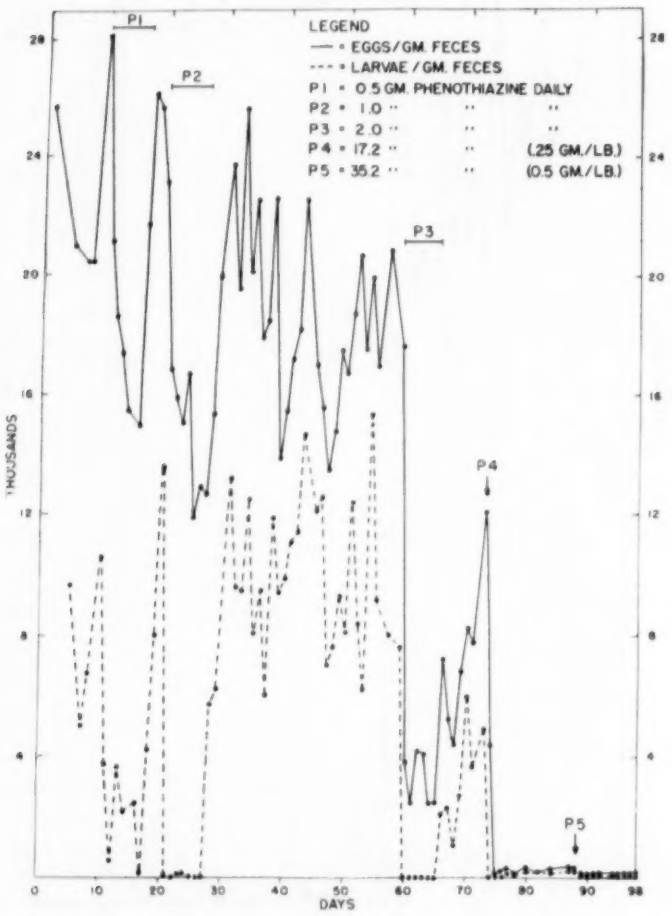

Fig. 7—Egg and larval counts on lamb 4T (experiment 2) infected with strain B mixed nematodes.

TABLE 1—Nematodes Recovered from Lambs in Experiment 1

Species	No. of worms			
	Strain A		Strain B	
	Lamb 48A	Lamb 48B	Lamb 410A	Lamb 410B
Haemonchus contortus	0	120	119	255
Ostertagia spp.	0	0	10	50
Trichostrongylus axei	10	7	40	47
Trichostrongylus colubriformis	0	0	30	90
Cooperia curticei	0	10	60	160
Chabertia ovina	0	0	71	57
Oesophagostomum columbianum	2	9	0	0
Oesophagostomum venulosum	0	0	7	14

ment recovery periods in these lambs corresponded with the return of the eggs.

In contrast with the foregoing, only a moderate reduction in e.p.g. was detected in strain B-infected lambs 4T (fig. 7) and 4X (fig. 8) with the seven-day course of treatment at the 0.5-Gm. per day level. The reduction during treatment averaged only 18.0 per cent, with a tendency to return to pretreatment levels during the post-treatment period. At the dose rate of 1.0 Gm. per day, a greater depression (42.0%) of the e.p.g. averages occurred during medication; however, the post-treatment recovery tendency was retained. A more complete (83.0%) egg count depression occurred when lamb 4T (fig. 7)

TABLE 2—Nematodes Recovered from Lamb in Experiment 2

Species	No. of worms			
	Strain A		Strain B	
	Lamb 4H	Lamb 4W	Lamb 4X	Lamb 4T
Haemonchus contortus	5	2	60	33
Trichostrongylus axei	7	0	0	0
Trichostrongylus colubriformis	0	0	0	7
Cooperia curticei	0	0	20	0
Oesophagostomum columbianum	1	60	0	0

was given 2.0 Gm. daily, with only a partial rebound after treatment.

Therapeutic dosing (0.25 Gm./lb.) of lambs 4T and 4X effectively reduced the average e.p.g. during the week after treatment to 7.2 per cent of the pretreatment average. When a second, heavier therapeutic dose (0.5 Gm./lb.) was given to these lambs, the e.p.g. was further reduced, but the percentage of reduction averaged less (75.0%). Low-grade counts persisted until necropsy of lamb 4X on the eightieth day and lamb 4T on the ninety-eighth day.

The inhibition of larval development, in strain B-infected lambs, during the 0.5-Gm. courses of treatment, was only 74.0 per cent in lamb 4T (fig. 7) and 97.0 per cent in lamb 4X (fig. 8); however, it was practically complete during the 1.0- and 2.0-Gm. courses of treatment. Rapid post-treatment recovery of larval development was characteristic and corresponded with the rises in the e.p.g. Following the therapeutic doses, no larval development was evident until the 72-hour sampling.

The number of worms recovered at necropsy from the lambs in experiment 2 is shown (table 2). Of the 7 *H. contortus* of strain A, 6 were males and the single female (from 4W) was devoid of eggs *in utero*. Recovery of the relatively large number of nodular worms from 4W was surprising in view of the preceding fecal examinations, and only 3 of the 20 females were devoid of eggs *in utero*. In comparison, more *H. contortus* were found in the strain B lambs, even though these lambs were subjected to much more rigorous treatment. Sex differentiation of these revealed a predominance of males, 46 in lamb 4X and 27 in lamb 4T.

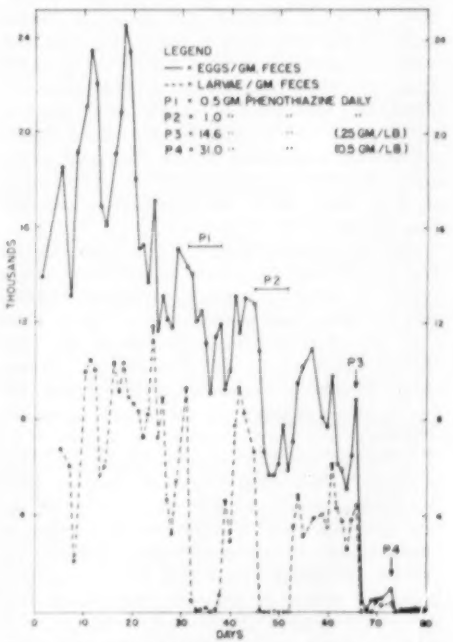

Fig. 8—Egg and larval counts on lamb 4X (experiment 2) infected with strain B mixed nematodes.

All of these female *H. contortus* showed eggs *in utero*.

Only female *H. contortus* of strain B were available for classification by their type of vulval processes. From lamb 4T, 3 and 2 were types B and F, respectively, and from lamb 4X, 1, 6, 1, and 6 were types A, B, D, and F, respectively.

DISCUSSION

Although the experimental procedures and treatments were identical, the responses of nematodes of strains A and B to the action of phenothiazine were remarkably consistent and strikingly different. More distinct differences between the strains were evident in the small daily dose trials than with the large therapeutic doses. Additional therapeutic trials, including a range of lower dose levels, are indicated.

Haemonchus contortus appeared to be the predominant species in both strains. This was evident from the worms collected from the control lambs in experiment 1 and the recognized prolific egg production capacity of *H. contortus*. Thus, the persistency of e.p.g. during treatment of lambs infected with strain B is difficult to explain except on the basis of a relatively greater drug tolerance than strain A. On the other hand, the low level of larval development which persisted during treatment of strain B could be accounted for by the drug tolerance of other species present. Additional studies have been made which elucidate this point.[5]

In both strains A and B, the bulk of the *H. contortus* females had sheep-strain types of vulval processes, although in both strains the percentages were lower than that reported as characteristic of *H. contortus* from sheep in Australia.[19] From these limited observations, differences between these strains on this basis were not evident.

Previous observations by Shorb and Haberman [22] indicated that 0.5 Gm. of phenothiazine daily would completely arrest the development of roundworm larvae in sheep feces. This was the characteristic response of strain A in the present study; however, strain B showed appreciable larval development during medication with 0.5-Gm. doses and inhibition was not complete below the 1.0-Gm. level.

Marked depression (81.5%) in *H. contortus* fecal egg counts, recorded by Page,[15] from 0.5-Gm. daily doses of phenothiazine in lambs, is comparable to strain A of the present study; in contrast, 2.0-Gm. daily doses were required to effect a similar degree of e.p.g. depression on strain B.

From Australia, Gordon [8] reported that 1.0- and 2.0-Gm. doses for five days were effective against *H. contortus* and *Oesophagostomum columbianum*. In this study, 1.0-Gm. doses for seven days in strain A lambs produced marked and apparently permanent changes, whereas 2.0 Gm. for six days in strain B lambs produced less marked changes which were only transient.

Drenching at the generally recommended therapeutic dose level (0.25 Gm./lb.) was effective in two tests against strain A, but much less effective in one of three tests at 0.25 Gm./lb. and two tests at 0.5 Gm./lb. against strain B. Survival of appreciable numbers of *H. contortus* at the latter dose was noteworthy, particularly in view of the reports [1, 9, 12] that doses on the order of 0.1 Gm./lb. would almost invariably remove all *H. contortus* from adult sheep.

On the basis of all four of the foregoing criteria, strain B appeared to be more drug tolerant than strain A and also more tolerant than nematodes previously studied by others as cited. This suggests that the more or less continuous contact of strain B with the drug over the extended period resulted in the selection or development of nematodes possessing a relatively high threshold to the action of phenothiazine. Therefore, somewhat less than effective control of strain B could be expected from medication under field conditions because of the additional variable factors encountered. However, these observations are not regarded as sufficient evidence to justify excluding the use of phenothiazine-salt mixes as prophylactics even with strain B.

Although the existence or eventual appearance of strains of nematodes possessing equal or greater tolerance to phenothiazine than strain B is suggested, the present investigations are limited in scope and the general picture should be clarified by additional controlled studies. Until otherwise established, strain B should be regarded as exceptional and the several other factors that may account for an ap-

parent failure or lack of effectiveness of phenothiazine or any anthelmintic should not be overlooked.

SUMMARY

A comparison was made of the action of phenothiazine on two strains of sheep nematodes that were selected on the basis of flock history as representative of minimum (strain A) and maximum (strain B) contact with this drug. Treatment of experimentally infected lambs with small daily doses resulted in consistent and strikingly different responses between these strains. Thus, 0.5 Gm. of phenothiazine daily completely inhibited larval development and depressed egg counts by 65 per cent in lambs infected with strain A; in contrast, 1.0 Gm. and 2.0 Gm. daily were required for these same effects in lambs infected with strain B. Limited experience with therapeutic doses also indicated a lack of effectiveness against strain B nematodes which not only had a higher threshold to the drug than strain A but also appeared more tolerant than strains of sheep nematodes studied previously by other investigators. The present observations are in accord with the strain B flock history which indicated a lack of effectiveness of phenothiazine under field conditions. These observations suggest that the prolonged use of phenothiazine in the flock has resulted in the development or selection of drug-resistant strains of nematodes possessing a significant level of tolerance to phenothiazine.

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Plasma Iron and Iron-Binding Capacity in Lambs

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As the result of the discovery of an iron-binding component in plasma,[1] the significance of plasma iron and plasma iron-binding capacity has been given renewed attention. A detailed discussion of the relationship of plasma iron to metabolism is beyond the scope of this paper; however, excellent reports are available in the literature.[2-7]

Plasma iron is not present in the ionized state, but rather as a complex with a protein fraction known as "siderophilin," "transferrin," or the *beta*-1 metal-combining protein.[1,8-10] As the name indicates, this is a *beta*-1 globulin and it is estimated[9] that it makes up approximately 3 per cent of the total serum protein in man.

The amount of transferrin present is not generally expressed in terms of protein levels but, rather, in terms of the amount of iron which may be bound by a given volume of plasma or serum, usually in micrograms per 100 ml. Transferrin *in vitro* may bind up to 1.25 μg. of iron per milligram[9]; however, *in vivo* in the normal man, it is only about one third saturated.[2,3,5-7,11,12] The iron-binding capacity of plasma has, therefore, been divided into two fractions; Laurell[6] has designated that part which is bound as the manifest iron-binding capacity (MIBC), and that part which is unbound as the latent iron-binding capacity (LIBC). The former is equal to the actual plasma or serum iron level. The sum of these two components may be referred to as either the saturation limit of serum for iron[6] or as the total iron-binding capacity (TIBC) of plasma or serum.

In man, it has been demonstrated that there is a marked diurnal fluctuation in plasma iron levels.[13] This fluctuation appears to be correlated with sleep and activity, but not with the degree of activity. The higher plasma iron values are associated with sleep. It has also been found that men have a higher MIBC and TIBC than do women.[11]

The study reported in this paper was undertaken with three objectives: (1) to determine the normal MIBC, LIBC, and TIBC of lamb plasma; (2) to determine whether or not there is a diurnal fluctuation in the MIBC; and (3) to determine if the iron-binding components in wether and ewe lambs are significantly different.

Methods

Laboratory.—In order to evaluate the plasma iron-binding components it is necessary to make two determinations, namely, the LIBC and the MIBC (plasma iron level). In this study, the method of Rath and Finch[14] was employed for the former determination.

The majority of published procedures for the determination of plasma iron are based on the same principles. The iron is first released from its complex with transferrin by acidification. Plasma proteins are then removed and, by addition of a suitable reducing agent, the iron is reduced to the ferrous state. The iron is then determined colorimetrically after forming complexes with such reagents as thiocyanate, *o*-phenanthroline, or 2:2′-dipyridyl.

The procedure used in this study was the result of modification and combination of existing methods[14-18] as follows:

1) All glassware was washed in dichromate cleaning solution followed by concentrated nitric acid and then placed in 2.5 per cent HCl for 24 hours. Prior to use, it was rinsed in iron-free distilled water and dried.

2) Two milliliters of plasma (or serum) were pipetted into 15.0-ml. centrifuge tubes.

3) One milliliter of 3.2 per cent HCl was added, mixed, and allowed to stand 30 minutes.

4) The mixture was then placed in a 90° C. water bath for five minutes.

5) After cooling, 2.0 ml. of 40.0 per cent trichloroacetic acid (TCA) was added and the mixture stirred.

6) The mixture was then placed in a 90° C. water bath for ten minutes.

7) After cooling, 1.0 ml. of iron-free distilled water was added and the mixture stirred and centrifuged.

8) Four milliliters of the supernatant were then transferred to a fresh 15.0-ml. tube.

9) One milliliter of 3.5 M NH_4OH was then added to the second tube.

10) Five milliliters of composite reagent[16] were

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This work was undertaken as a contributing project to the Western Regional Project, "Internal Parasites of Ruminants."

then added to the second tube and, after mixing, the tube was placed in a 90 C. water bath for ten minutes.

11) A suitable amount of the resulting mixture was placed in cuvettes and the optical density determined in a Coleman spectrophotometer at a wave length of 520 mμ. (If the optical density reads less than 0.05, it is advisable to determine the value on a duplicate sample after adding a known amount of iron.)

12) A reagent blank was prepared by using 2.0 ml. of iron-free distilled water as a substitute for plasma.

13) A standard curve was prepared by making proper dilutions of a stock solution prepared from iron wire.

The accuracy of this procedure was determined in the following manner: Samples of blood sufficient to allow removal of 4.0 ml. of plasma were obtained in heparinized syringes from sheep. The plasma was divided into two 2.0-ml. samples. The equivalent of 150 μg. of iron/100 ml. was added to one of the samples and the iron levels determined for both of them. The accuracy was then calculated from the amount of added iron recovered.

Animals.—The lambs were removed from their dams on the first day following birth. They were then fed cows' milk until 2 weeks of age. At this time, the milk was supplemented with chopped alfalfa hay and a mineral-concentrate mixture made up as follows: crude protein, not less than 12.0 per cent; crude fat, not less than 2.0 per cent; crude fiber, not more than 7.5 per cent; and ash and added minerals, not more than 8.0 per cent.

The ingredients were ground barley, rolled barley, wheat, standard middlings, wheat bran, alfalfa meal, meat and bone scraps, soybean oil meal, coconut oil meal, degossypolized cottonseed meal, wheat germ, condensed fish solubles, ground milo, ground yellow corn, cane molasses, linseed oil meal, dried whole whey, irradiated yeast, vitamin A oil, vitamin B_{12} and antibiotic feed supplement, riboflavin, niacin, calcium, pantothenate, choline chloride, ground limestone, and mineralized salt containing iron oxide, manganese sulfate, sodium thiosulfate, copper carbonate, cobalt carbonate, potassium iodide, and dicalcium phosphate.

The amount of milk fed was increased from approximately 5.0 per cent of body weight at birth to approximately 15.0 per cent at 2 weeks of age. The mineral-concentrate supplement, as well as chopped alfalfa hay, was fed free choice.

The lambs were weaned at 3 months of age, at which time the ration was confined to chopped alfalfa hay and 0.5 oz. of salt. Although some of the lambs shed small numbers of coccidial oocysts, they remained relatively free of helminths.

In the first trial, 5 wether and 5 ewe lambs were employed. Beginning at 6:00 a.m., blood samples were obtained every six hours for 48 hours (table 2). This trial was conducted in August, 1955, when the lambs were 8 months old. Both the MIBC and LIBC were determined at each sampling.

In a second experiment, only wether lambs, 12 months old, were used. This time, the MIBC was determined for each sample, but the LIBC was determined only for the first and last samples.

Statistical Methods.—The standard deviation was found by the formula $\sigma = \frac{\sqrt{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}}{n-1}$, while the formula $\sigma m = \frac{\sigma}{\sqrt{n-1}}$ was used for determining the standard error of the mean.

The mean value found for the TIBC of individual animals was used for the purpose of calculating the percentage of saturation (100 · MIBC/TIBC).

RESULTS

Analysis of data from 18 individual plasma samples, relative to the accuracy of the procedure for determining plasma iron levels, revealed the following: a mean recovery of 148.5 μg./100 ml., representing 99.0 per cent of the added iron; a standard deviation of 11.0 μg./100 ml. or 7.3 per cent of the added iron; and a standard error of the mean of 2.7 μg./100 ml. or 1.8 per cent of the added iron. The fact that these data were collected over a period of three months lends support to the conclusion that the procedure is sufficiently accurate and reliable.

No specific data were obtained relative to the accuracy of the method used in determining the LIBC [11]; however, analysis of data from the lamb showing the greatest fluctuation in TIBC showed a standard deviation of 25.0 μg./100 ml. and a standard error of 10.0 μg./100 ml. Since fluctuations in TIBC over a period as short as 48 hours would be largely due to errors in procedures used for determining both the MIBC and the LIBC, it is apparent that the latter procedure was also sufficiently accurate.

Data are presented on the mean values for plasma iron-binding components of ewe and wether lambs 8 months old (table 1; fig. 1) and on these same lambs at six-hour intervals during the 48-hour period (table 2; fig. 1).

Analysis of the data on individual wether lambs revealed a mean fluctuation in plasma iron (lowest to highest MIBC) of 75.0 μg./100 ml. with a standard deviation of 20.0 μg./100 ml. and a standard error of 10.0 μg./100 ml. Mean fluctuation of individual ewe lambs was 56.0 μg./100 ml. with a standard deviation of 14.0 μg./

TABLE 1—Mean Values for Plasma Iron-Binding Components in Wether and Ewe Lambs 8 Months Old

	Manifest iron-binding capacity (μg./100 ml.)	Latent iron-binding capacity (μg./100 ml.)	Total iron-binding capacity (μg./100 ml.)	Percentage of saturation (%)
Wether	131±7*	196±14	327±11	40±3
Ewe	135±4	204±8	339±6	39±3

* Standard error of the mean.

100 ml. and a standard error of 7.0 μg./100 ml.

The percentage of saturation ranged from 26.0 to 59.0 per cent in the wether lambs and 24.0 to 54.0 per cent in the ewe lambs. The standard deviation was 7.0 per cent and 6.0 per cent, respectively.

The data collected on the same wether lambs when 1 year of age are summarized (tables 3 and 4; fig. 2).

Analysis of individual data from lambs 12 months old showed a mean plasma iron fluctuation for the 24-hour period of 60.0 μg./100 ml., with a standard deviation of 17.0 μg./100 ml., and a standard error of 9.0 μg./100 ml. The percentage of saturation ranged from 44.0 to 74.0 per cent with a standard deviation of 11.0 per cent.

DISCUSSION

Data (table 1; fig. 1) indicate that there were no significant differences between the plasma iron and plasma iron-binding capacity of wether and ewe lambs 8 months old.

An increase in the mean plasma iron level (MIBC), from 131.0 μg./100 ml. to 193.0 μg./100 ml., between the ages of 8 and 12 months occurred in the wether lambs (tables 1 and 3; fig. 1 and 2). There was no significant change in the TIBC during this same period.

The MIBC, LIBC, and TIBC of lambs 8 months old were comparable to that of man.[5] The values found for wether lambs 12 months old, with the exception of the TIBC, were quite different. Ramsay,[14] using his method for the determination of plasma iron, has reported values in man to be some 30 to 40 per cent higher than those reported by other workers.[15,16] He also reported that in the case of a mature ewe

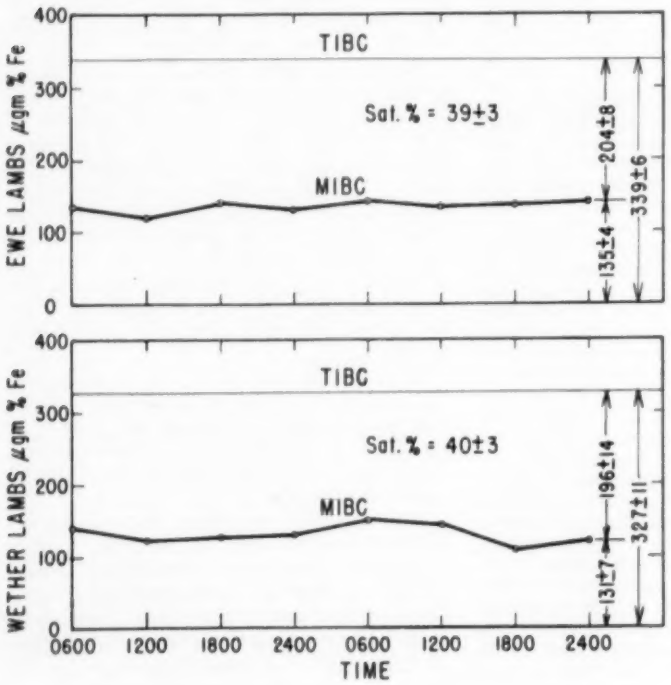

Fig. 1—Fluctuations in the plasma iron and iron-binding capacity of wether and ewe lambs 8 months of age.

TABLE 2—Mean Plasma Iron Values for Wether and Ewe Lambs 8 Months Old at Six-Hour Intervals During a 48-Hour Period

	6 a.m.	12 a.m.	6 p.m.	12 p.m.	6 a.m.	12 a.m.	6 p.m.	12 p.m.
Wethers	140±11*	124±10	128±6	132±15	150±10	144±10	108±9	122±13
Ewes	135±9	120±9	139±3	130±7	141±8	135±4	137±6	142±12

* Standard error of the mean.

he had found a plasma iron level of 300.0 μg./100 ml. This latter value exceeds that found by the present writers in year-old wether lambs by approximately 33 per cent. If it can be assumed that his procedure for determining plasma iron results in values 30 to 40 per cent higher than the procedure used in this work, it would appear that year-old wether lambs have plasma iron levels comparable to the adult ewe examined by Ramsey.

TABLE 3—Mean Values for Plasma Iron-Binding Components in Wether Lambs 12 Months Old

Manifest iron-binding capacity	Latent iron-binding capacity	Total iron-binding capacity	Percentage of saturation
193±7	141±10	334±18	58±3

Data from ewe lambs (table 2; fig. 1), while showing fluctuation in the plasma iron level, do not indicate that the fluctuation was of a diurnal nature. In the case of the wether lambs (tables 2 and 4; fig. 1 and 2), there is some indication of a diurnal fluctuation, a minimum value occurring between 12:00 a.m. and 12:00 p.m. and a maximum value between 12:00 p.m. and 12:00 a.m. This periodicity would be comparable to that in man.[13] Some doubt as to this being a true diurnal variation is encountered when it is noted that with one minor exception (wether lambs 8 months old) all of the minimal values were found six hours following the first sampling. In this respect, it has been demonstrated[17] that injection of adrenalin,[®] adrenocortical extract, or adrenocorticotrophic hormone into dogs produces marked hypoferremia which becomes maximal in six to eight hours following injection. The apparent diurnal fluctuation of wether lambs may, therefore, be no more than the result of hyperactivity of the adrenal gland resulting from excitement brought about during the venipuncture procedure. The fact that the same reduction in plasma iron occurred on the second day of the first experiment, however, would lend support to the possible occurrence of a diurnal variation. It may tentatively be concluded that while lambs may possibly show diurnal fluctuation in plasma iron levels, this is not sufficiently established to warrant dependency on it in experimental work.

TABLE 4—Mean Plasma Iron Levels in Wether Lambs 12 Months Old at Six-Hour Intervals During a 24-Hour Period

12 a.m.	6 p.m.	12 p.m.	6 a.m.
227±9	165±8	178±8	202±15

It is not, as yet, possible to establish definitive limits of normality in so far as plasma iron and iron-binding capacity in lambs is concerned. It would appear, how-

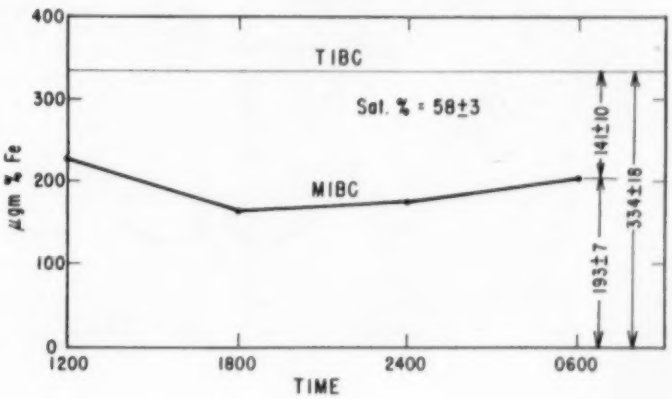

Fig. 2—Fluctuations in the plasma iron and iron-binding capacity of wether lambs 12 months of age.

ever, that a TIBC of 290.0 to 370.0 μg./100 ml. might well encompass the normal for these animals.

In 95 per cent of 8-month-old lambs, comparable to those used in these experiments, the saturation percentage would be expected to fall between 27 per cent and 53 per cent, and when 12 months old, between 36 per cent and 80 per cent.

If it is assumed that in its ability to bind iron, ovine transferrin is similar to that of man, it may then be calculated from the work of Surgenor *et al.*[9] that transferrin is present in the amount of 260.0 to 270.0 mg./ml. in lamb plasma and serum. This would correspond to 4 to 5 per cent of the total serum protein in the case of the lambs used in the present study.

SUMMARY

The plasma iron and iron-binding capacity of ewe and wether lambs, 8 months old, did not differ significantly under the conditions of this experiment.

The plasma iron levels (MIBC) of wether lambs increased from a mean of 133 to 193 μg./100 ml. between the ages of 8 and 12 months. Since no change occurred in the total iron-binding capacity (TIBC), this increase in plasma iron resulted in an increase in the percentage of saturation, of transferrin with iron, from a mean of 40 to 58 per cent.

It is tentatively concluded that lambs may possibly show diurnal fluctuation in plasma iron levels when undisturbed, however, this can not be depended upon when they are maintained under experimental conditions.

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Studies on the Mechanism of Infection of the Brain with *Listeria Monocytogenes*

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LISTERIOSIS has been found in many animal species and man. The lesions most commonly observed are purulent encephalitis and focal necrosis of parenchymatous organs. Encephalitic listeriosis, particularly in sheep and goats, is almost invariably fatal. Encephalitis has been produced experimentally in mice, rabbits, pigs, and sheep by inoculating *Listeria monocytogenes* intranasally or into the conjunctival sac.[1-5] However, the pathway by which *L. monocytogenes* placed on the nasal mucous membrane or in the conjunctival sac reached the brain to produce encephalitis was not determined. Pallaske [3] assumed that infection of the brain might take place via the olfactory nerve. However, the fact that lesions in the central nervous system of affected animals are confined almost exclusively to the brain stem contradicts this hypothesis. Burn [6] suggested that the brain might be infected from the blood stream. Olson *et al.*[7] and Olson [8] suggested that a virus might act in collaboration with *L. monocytogenes* to produce encephalitis.

The pathway by which *L. monocytogenes* might reach the brain was investigated by the present authors. These studies have continued from the time they first isolated *L. monocytogenes* from goats with encephalitis in Japan.[9] Clinical, bacteriological, and histopathological observations of naturally and artificially infected animals suggested that *L. monocytogenes* might invade the brain by way of the trigeminal nerve. Further support for this hypothesis has been obtained from experiments in which encephalitis was readily produced in rabbits, mice, and goats by inoculating *L. monocytogenes* in such a manner that the bacterium might be spread through the trigeminal nerve. Some of these studies have been published in a series of preliminary reports written in Japanese.[10-13] The present communication presents the data accumulated in these studies.

MATERIALS AND METHODS

A strain of *L. monocytogenes* designated "511," isolated by Asahi *et al.*[9] in January, 1951, from the brain of a goat, was employed. It was cultivated on liver infusion agar slants at 37 C. for 24 hours. For inoculation, the culture was suspended in saline to a concentration of 1 mg. of growth per milliliter of saline.

The following three routes of exposure were employed:

Conjunctival Instillation.—One or 2 drops of bacterial suspension were instilled into the conjunctival sac of goats and rabbits.

Inoculation into the Lip.—Minute wounds were made in mice, rabbits, and goats by puncturing the lip or other oral mucous membranes 20 to 30 times with a hypodermic needle 0.25 mm. in diameter. The wounds were so superficial that no bleeding occurred. Immediately thereafter, the bacterial suspension was placed on the prepared area. This site was selected for inoculation since numerous branches of the trigeminal nerve are found in this part of the body.

Feeding.—An arbitrarily selected amount of bacterial suspension was mixed with small fish

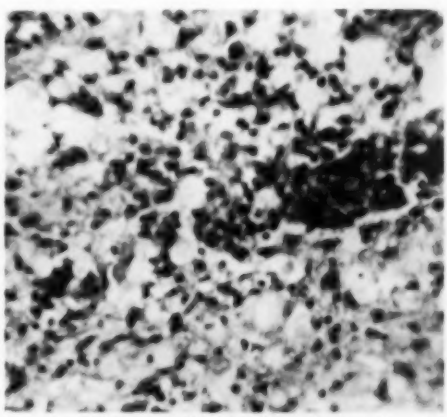

Fig. 1—Glial cell infiltration in medulla oblongata of goat 13 (7 in fig. 2). x 300.

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dried after boiling (Niboshi) and bean shells. These were fed to mice and goats, respectively, and are so rough that minute invisible wounds may be made in the mouth when they are eaten. This was given at a rate of 2 Gm. once a day per mouse, and 1 kg. twice a day per goat.

Tissues for histological examination from animals which died or were killed were fixed in 10 per cent formalin and stained either with hematoxylin-eosin or Weigert's stain for bacteria in tissues. Lesions in the tissue sections of artificially infected animals were compared with those from 5 naturally infected goats and 2 naturally infected sheep.

RESULTS

Conjunctival Instillation of Goats.—One animal, goat 13, a Saanen, 3 to 4 months old, developed encephalitis similar to that seen in natural infections. The body temperature rose to 105.4 F. on the day following instillation and remained fairly constant, with only 1.0 to 2.0 F. fluctuation for about two weeks. The temperature then dropped to its normal level of 103.1. On postexposure days 5 to 7, the animal was listless and slight diarrhea was observed. The same clinical symptoms appeared again on days 11 to 14. The conjunctiva showed no abnormality. A high leukocyte count (maximum, 19,000 to 21,000/mm.^{3}) was observed on days 1 to 4 and 8 to 14. On postexposure day 20, the animal was killed by exsanguination. Macroscopic examination revealed no changes in the central nervous system or other organs.

Histologically, a small lesion consisting of glial cells and mononuclear leukocytes was found in the brain stem (fig. 1). No lesions were seen in the other organs. The lesion in the brain stem was localized in the region of the spinal tract of the trigeminal nerve from the pons to somewhat below the root of the hypoglossal nerve (fig. 2). Infiltrations of mononuclear leukocytes also were found in the left trigeminal nerve, particularly in the endo-

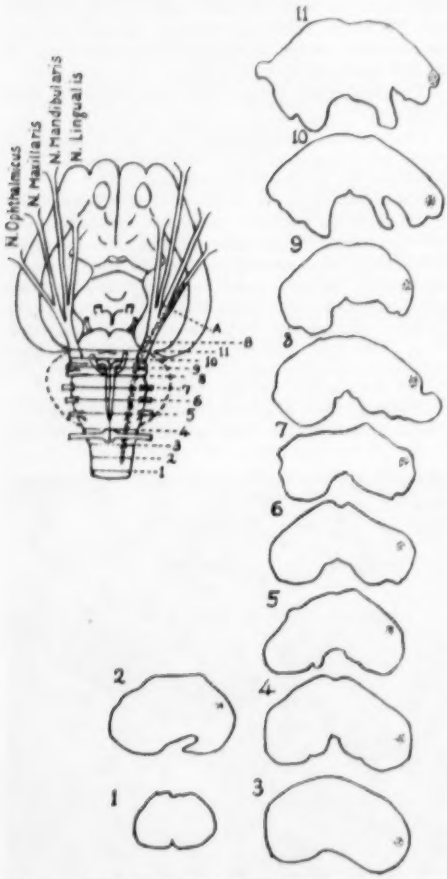


Fig. 2—Distribution of lesions in the brain of goat 13 exposed by conjunctival instillation.

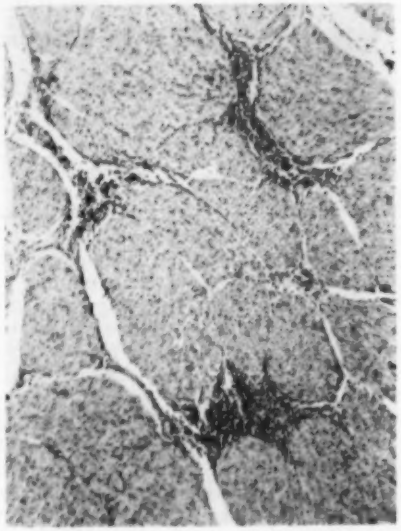


Fig. 3—Mononuclear round cell infiltration of the root of the trigeminal nerve from goat 13 exposed by conjunctival instillation. x 78.

neurium from the root to the branch of the ophthalmic nerve (fig. 1, 2, and 3). This finding coincided with the fact that the animal had been inoculated in the left conjunctival sac. *Listeria monocytogenes* was isolated from these lesions but not from any other organs, including the medulla oblongata.

TABLE 1—Artificial Infection of Mice by Inoculation in Various Sites Where the Trigeminal Nerve Was Distributed

Route of inoculation	No. of mice tested	Encephalitis	Septicemia	No symptoms
Nasal mucous membrane	15	9 (60%)	2	4
Oral mucous membrane				
Lip	10	9 (90%)	0	1
Gum	10	6 (60%)	3	1
Parotid	10	6 (60%)	3	1
Oral cavity	10	8 (80%)	1	1
Tongue	10	1 (10%)	9	0

Conjunctival Instillation of Rabbits.—Fever was not observed in any of the 4 animals after exposure. On postinstillation days 2 to 3, the conjunctiva was congested and swollen. There was excessive lacrimation; the eyes were sensitive to light and discharged a thick purulent exudate. By day 5, the conjunctiva was thickened by edema, so that the animals could not open their eyes. The condition then gradually improved and, by day 10, they were able to keep their eyes open. During this period, the leukocyte count showed frequent irregular rises. In animals 1, 2, and 4, the monocyte count gradually increased, especially in animal 2, the count being 23.9 per cent at the maximum. Fifteen days after inoculation, the animals were killed by exsanguination.

At necropsy, rabbits 1, 2, and 4 showed conjunctival congestion and corneal turbidity in the eyes which were exposed. In all rabbits, the liver had five to eight small, solid, grayish white nodules. The other organs appeared normal. The cerebrum and spinal cord showed no changes. Histological investigation of rabbits 1 and 4 revealed no changes in the visceral organs other than some Eimeria nodules in the liver. Only rabbit 4 showed detectable alterations in the cerebrum and trigeminal nerve. These consisted of glial cell infiltration along the spinal tract of the trigeminal nerve, beginning at the corpus rhomboideus and continuing to the posterior part of the medulla oblongata. The infiltrated areas showed a spongelike appearance and were surrounded by perivascular cell infiltrations. In the trigeminal nerve, cell infiltrations were observed in the neurium or between the nerve fibers. No changes were seen in the maxillary nerve. Recovery of *L. monocytogenes* was attempted from the heart blood, lung, liver, spleen, kidney, cerebrum, and conjunctiva with negative results.

Inoculation of Mice.—The lip, gum, parotid region, oral cavity, and tongue were selected for inoculation. Ten mice were used for each of the inoculation sites. The nasal mucous membrane was also included as the site of inoculation in 15 mice (table 1). Some mice died on postinoculation days 1 to 5. They showed depression, anorexia, ruffled fur, trembling, diarrhea, and conjunctivitis, but no nervous signs. In contrast, mice which became ill on or after day 6 demonstrated circling movements (when stimuli were applied by finger to the circling mice, they began to roll), paralysis in all legs or only the hindlegs, and excessive salivation (fig. 4). Most of these animals died one or two days after the appearance of signs of encephalitic lis-

Fig. 4—Mice showing symptoms of encephalitic listeriosis.
Left to right: circling movement; rolling of the body; wet face due to excessive salivation.

teriosis similar to those observed in natural infections of sheep and goats.

Histopathologically, the quickly fatal cases were characterized by focal necrosis of the visceral organs such as the liver, spleen, and kidney. No lesions were found in the brain but *L. monocytogenes* was isolated from the visceral organs and blood, suggesting a septicemia. In mice which showed nervous signs, lesions were found in the brain stem (fig. 5). These were characterized by infiltration of neutrophilic leukocytes, glial cells, and perivascular cell infiltration. These lesions were similar to those observed in natural cases of encephalitic listeriosis of sheep and goats. In addition, there were a few cases of a "mixed" type which showed both cerebral lesions and focal necrosis in the visceral organs. However, in all cases of this type mononuclear round cell infiltrations were always observed. *Listeria monocytogenes* was isolated only from the brain stem in most animals showing nervous signs but, in the so-called mixed type, the organism could also be isolated from the visceral organs. These methods of inoculation produced encephalitis in a high percentage of the exposed mice (table 1). The lip seemed to be the most effective route of inoculation.

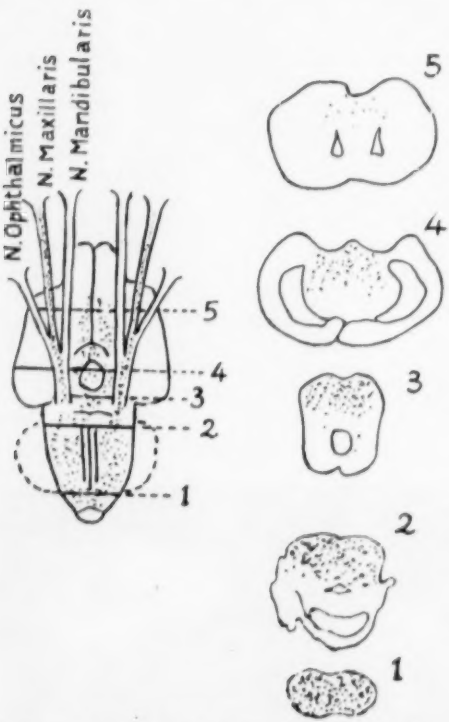


Fig. 5—Distribution of lesions in the brain of a mouse infected through minute wounds made in the lip.

In another series of experiments, 39 mice were exposed through the lip. Three to 7 mice were killed every day from 24 hours to eight days after inoculation. The trigeminal nerve, brain, and other organs were examined bacteriologically and histologically.

The 14 mice killed during the first three postinoculation days showed no signs of illness. On day 4, 1 of the 4 mice killed showed torticollis and unusual movements of the hindlegs. On day 6, 4 of the 7 killed mice showed torticollis, salivation, trembling of the body, rolling movement, and paralysis of the hindlegs. Half of the 6 mice killed on day 7 and all 5 on day 8 showed these nervous signs. The various tissues of exposed animals were cultured on liver infusion agar slants and in broth. *Listeria monocytogenes* was first isolated from 1 of the 3 mice destroyed on day 5. This isolation was made from the medulla oblongata. Thereafter, the rate of isolation increased gradually, *i.e.*, six out of seven on day 6 and five out of five on day 7. Isolation of *L. monocytogenes* from the cerebrum was unsuccessful throughout the observation period. Among the visceral organs of the 39 animals cultured, *L. monocytogenes* was isolated from the spleen of 6 and from the kidney of 1.

Where the organism had been inoculated in the lip tissues, rather marked infiltration consisting of both polymorphonuclear and mononuclear round cells was observed for eight days following inoculation. This observation correlated with the results of cultures of this tissue; *L. monocytogenes* being isolated from the lip of all but 3 of the 28 mice killed during the first six postexposure days. It also was isolated from the lip of 2 of the 6 mice killed on day 7 but not from any of the 5 mice killed on day 8. Mononuclear leukocytic infiltrations were found throughout the trigeminal nerve, from the minute branches distributed in the lips to the maxillary nerve and then back to the medulla oblongata. The lesion extended to the root of the trigeminal nerve in 1 of the 4 mice killed after 24 hours, in 2 of the 5

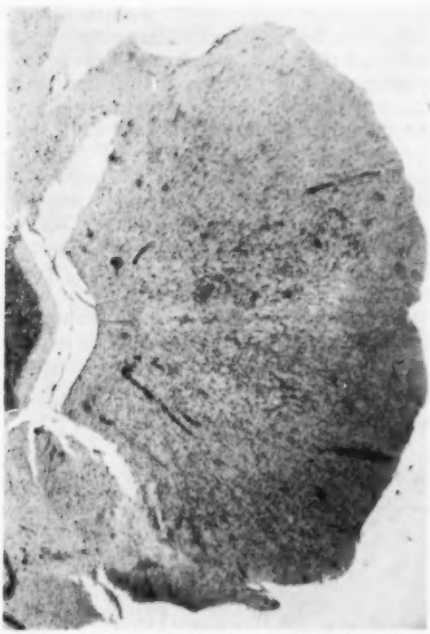


Fig. 6—Mononuclear round cell infiltration in the medulla oblongata of a mouse infected through minute wounds made in the lip. x 22.

after two days, and in 1 of the 5 after three days. Similar cellular infiltration was found in the medulla oblongata of 1 of the 4 mice killed on day 4 and in 1 of the 3 after day 5 (fig. 6). These mice developed slight nervous signs. Similar changes of the trigeminal nerve and medulla oblongata were found in all mice killed on postinoculation days 6 to 8. Of these, 1 showed no clinical and 3 no nervous signs when killed on days 7 and 8.

Inoculation into the Lip of Rabbits.—Six rabbits weighing 1.0 to 1.8 kg. were inoculated with the bacterial suspension through minute wounds in the mucous membrane of the upper lip. Congestion and swelling of the inoculated area were observed for several days after exposure. The animals were somewhat depressed but gradually recovered. One suddenly died on postinoculation day 5. None of the animals showed nervous signs. A remarkable increase of neutrophils in the blood was observed during the first four to five days after inoculation. The blood picture then gradually returned to normal. No monocytosis was observed.

These animals were killed for necropsy, animals 1 and 2 on day 17 and animals 3, 4, and 6 on day 27. No macroscopic lesions were observed either in the brain or visceral organs of any of the animals. Cultures prepared from the liver, spleen, kidney, heart blood, lung, cerebrum, and medulla oblongata failed to reveal *L. monocytogenes*. Nevertheless, infiltrations of mononuclear round cells, glial cells, and perivascular cell infiltrations were observed in the trigeminal nerve and brain stem in all animals except 5. These changes (fig. 7) occurred in the trigeminal nerve tract and extended to the medulla oblongata. The lesions were similar to those observed in animals inoculated by conjunctival instillation.

Inoculation into the Lip of Goats.—Eleven goats were inoculated with a relatively small quantity of culture (1/500 mg. or less). All showed a slight fever on postinoculation days 1 to 2 and then returned to normal. Seven remained normal after this initial fever subsided, while 4 experienced a second fever, ranging from 104.0 to 105.8 F. Two or 3 days after the onset

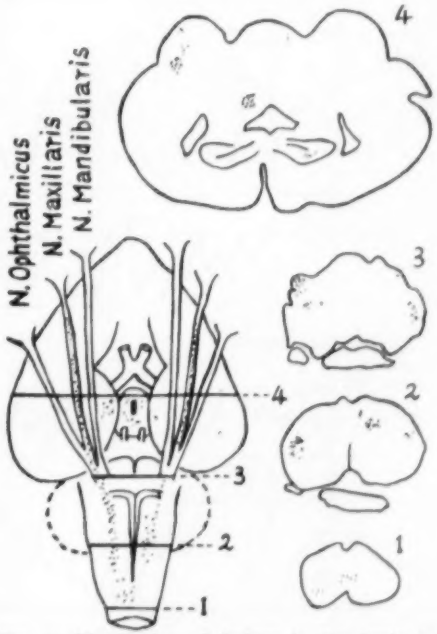


Fig. 7—Distribution of lesions in the brain of rabbit 1 infected through minute wounds made in the upper lip.

of the second fever and 17 to 28 days after inoculation, the animals demonstrated listlessness, rolling movement, torticollis, paralysis of the masseter muscles, and salivation. The animals quickly fell into stupor and coma and died in one or two days (fig. 8). The leukocyte count decreased soon after inoculation and then gradually returned to normal. Neutrophilia occurred as the nervous symptoms progressed.

At necropsy, no macroscopic abnormalities in the central nervous system or visceral organs were seen. Cultures were prepared from the cerebrum, medulla oblongata, spinal medulla, spinal fluid, liver, spleen, kidney, adrenal gland, pancreas, parotid gland, heart, lung, bone marrow, pulmonary lymph node, hepatic lymph node, renal lymph node, intestinal lymph node, nasal mucous membrane, oral mucous membrane, conjunctiva, urine, and feces. *Listeria monocytogenes* was isolated from the medulla oblongata in 3 of the 4 goats (animals 41, 43, and 45) and from the cervical spinal medulla in 2 (animals 43 and 45). All other cultures were negative.

Histological examination of the visceral organs showed no alterations. However, the brain and spinal cord demonstrated remarkable changes in all 4 goats which developed nervous signs. These lesions were essentially the same as observed in natural cases of listerellal encephalitis of sheep and goats. Goat 66 had a small lesion in the

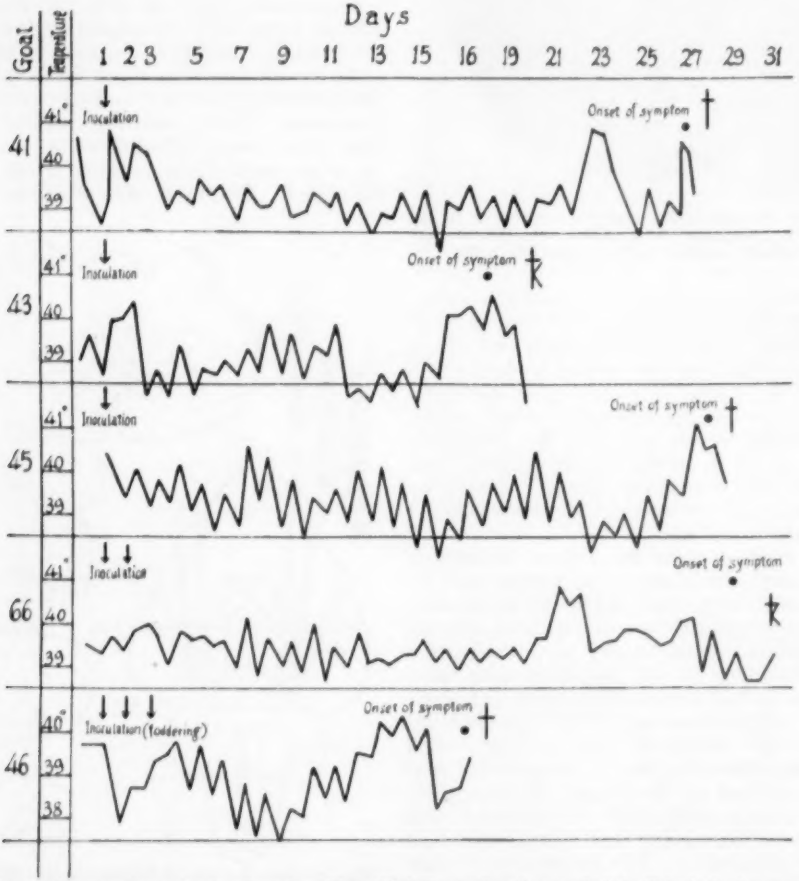


Fig. 8—Temperature charts of goats with artificially produced encephalitic listeriosis (exposed through punctured lips).

TABLE 2—Distribution of Lesions in the Central Nervous System of Goats with Artificially Induced Encephalitic Listeriosis (Exposed Through Punctured Lips)

Location	Goat No. 41		43		45		66		46		49	
	M*	P*	M	P	M	P	M	P	M	P	M	P
Brain												
Olfactory bulb	+	−	−	−	+	−	−	−	−	−	−	−
L. frontal	+	−	−	−	+	−	−	−	−	−	−	−
L. parietal	....	....	−	−	+	−	−	−	−	−	−	−
L. piriform	....	....	−	−	+	−	−	−	−	−	−	−
L. temporal	−	−	−	−	+	−	−	−	−	−	−	−
L. occipital	+	−	−	−	−	−	−	−	−	−	−	−
Corpus striatum	−	−	−	−	−	−	−	−	−	−	−	−
Thalamus	−	−	−	+	−	−	−	−	−	−	−	+
Cornu ammonis	+	−	−	−	−	+	−	−	−	−	−	−
Corpora quadrigemine	+	−	−	+	++	−	−	−	−	−	+	+
Cerebral peduncle	++	+++	−	++	+	++	−	+	−	+	+	+
Pons	++	+++	−	+++	++	+++	−	+	−	++	+	++
Cerebellum	+	+	+	++	++	++	−	−	+	+	+	+
Medulla oblongata	+	+++	+	+++	++	+++	−	+	+	+++	+++	++
Spinal cord												
Cervical	+	−	−	−	+	+	−	−	−	++	+++	+
Thoracic	−	−	−	−	−	−	−	−	−	−	−	−
Lumbar	−	−	−	−	−	−	−	−	−	−	−	−
Sacral	−	−	−	−	−	−	−	−	−	−	−	−

* Left and right columns under goat numbers refer to meningeal (M, left) and parenchymatous (P, right) lesions, respectively. The minus sign (−) indicates no lesion and the plus signs (+, ++, and +++) indicate the severity of the lesion.

meninges, while goats 41 and 43 showed meningeal lesions extending from the dorsal middle of the medulla oblongata to the root of the olfactory nerve. In general, lesions in the brain stem were of four distinct types. In the most severely affected areas, there were diffuse and focal infiltrations consisting predominantly of polymorphonuclear leukocytes. This occurred most commonly in the medulla oblongata and

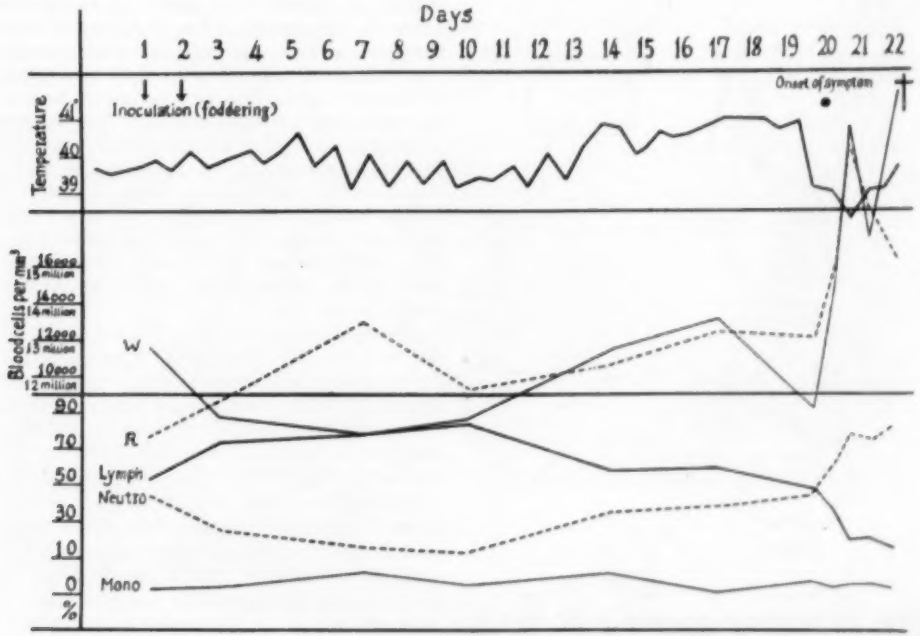


Fig. 9—Temperature chart and blood reaction of goat 49 infected by feeding a dried rough fish product.

pons. In other areas the infiltration consisted of both polymorphonuclear and glial cells. In goat 66 which showed only mild alterations, the predominant lesion was infiltration of glial cells with perivascular cuffing of mononuclear cells throughout the affected areas (table 2). These findings were similar to those observed in naturally infected animals. Mononuclear leukocytes were observed from the maxillary or mandibular nerves to the medulla oblongata through the root of the trigeminal nerve. These lesions simulated those obtained by inoculating mice through minute wounds. Other cerebral nerves such as the optic, glossopharyngeal, vagus, accessory, and hypoglossal were also found to be infiltrated with mononuclear cells. In contrast to the lesions in the trigeminal nerve, the lesions in these nerves were confined to within about 1 to 2 cm. from their roots.

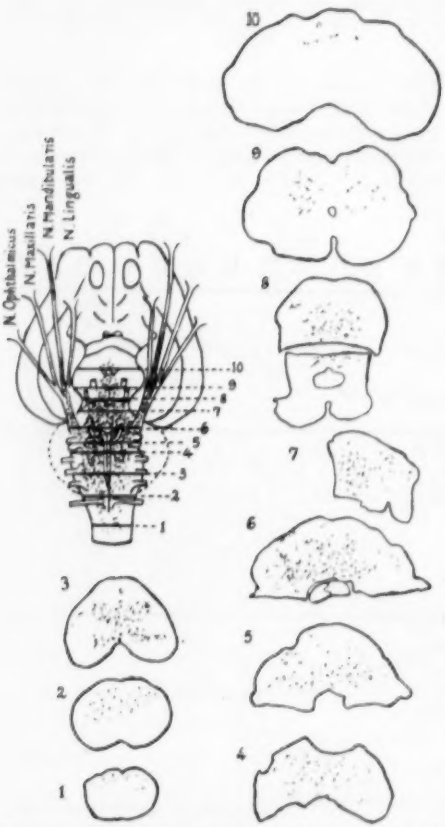


Fig. 10—Distribution of lesions in the brain of goat 46 infected by feeding a dried, rough fish product.

Feeding of Mice.[12]—Of 20 mice weighing 12 to 15 Gm. given daily 2 Gm. of Niboshi dipped in a suspension of *L. monocytogenes*, 13 developed typical nervous symptoms between postinoculation days 7 and 15. They showed salivation, circling movements, and incoordination of the extremities followed by coma and death. At necropsy, only a few macroscopic changes in the brain and visceral organs were found. However, histologically polymorphonuclear leukocytes in the brain stem and mononuclear leukocytes in or between the nerve fibers of the trigeminal nerve were observed. *Listeria monocytogenes* was isolated from the brain stem of all 13 mice which showed nervous signs.

Feeding of Goats.[13]—Bean shells contaminated with a suspension of *L. monocytogenes* were given once a day for two or three days to 8 male goats, 2 to 4 months old. Of these, 2 goats showed fever, 104.0 to 105.8 F. for five to six days prior to the onset of nervous signs which included dullness, circling, torticollis, paralysis of masseter muscles, salivation, drooping ear, and optical disturbances. These animals died within one to two days later. Immediately after the appearance of nervous signs, erythrocytosis and neutrophilic leukocytosis were observed (fig. 9). At necropsy, goat 49 showed thickening and yellowish brown discoloration of the medullar pia mater (aureomycin® had been injected into the cerebrospinal cavity for therapeutic trial) but no other macroscopic changes. Microscopic lesions were localized in the brain stem and along the trigeminal nerve (fig. 10). Mononuclear round cells were found both in the endoneurium and between the nerve fibers (fig. 11).

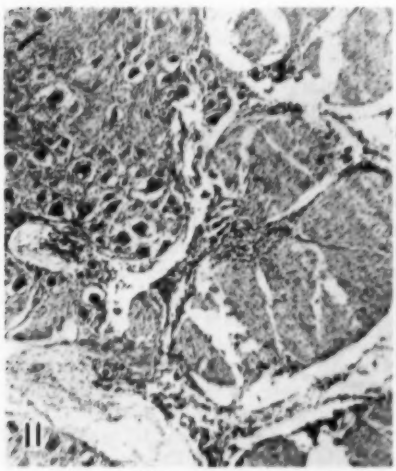
Histopathology of the Trigeminal and Other Cerebral Nerves of Naturally Infected Sheep and Goats.—Since the lesions appeared to be confined to the trigeminal nerve and its branches in mice, in rabbits, and in goats with artificially induced encephalitic listeriosis, detailed histological examinations were performed on tissues from 7 naturally infected animals—5 goats and 2 sheep. Four of these animals were studied extensively, but a complete set of sections was not available for the other 3 animals.

Some cellular reactions were found at

the root of either one or both trigeminal nerves of all the animals. The lesions were composed mainly of mononuclear round cell infiltrations in or between the nerve fibers (fig. 12). Neutrophilic cells were often seen in the lesions in the more severe cases. The same cell reactions were seen at the periphery of the maxillary, mandibular, and lingual nerves (fig. 13). A few gram-positive bacilli were seen in all 4 animals examined. In 2 animals, a goat and a sheep, portions of the other 11 cerebral nerves situated in the subarachnoid space showed some changes. These consisted of mononuclear round cell infiltrations in the olfactory nerve tract and in the facial and acoustic nerves of one goat, and in the oculomotor, facial, acoustic, accessory, and hypoglossal nerves of 1 sheep. These changes were limited to the portion of the nerves close to the brain, *viz.*: within 3 cm. (oculomotor), 2 cm. (facial), 2 cm. (glossopharyngeal), and 5 cm. (hypoglossal) from the nerve root.

DISCUSSION

Observations made by a number of investigators, as well as by the present authors, indicate that most natural cases of listeriosis in sheep and goats, in other countries as well as in Japan, are characterized by involvement of the central nervous system. The pathogenesis of this condition has been studied by many authors. Gill [1] presumed that *L. monocytogenes* might invade the brain by way of the nasal mucous membrane and the olfactory nerve. He attempted to infect sheep by intranasal instillation and succeeded in producing encephalitis. Similar results were obtained in mice by Jungherr [2] and in mice and rabbits by Pallaske.[3] Graham *et al.*[4] and Gray *et al.*[5] successfully produced encephalitic listeriosis in a pig and a rabbit, respectively, by instillation of the bacterium into the conjunctival sac. However, these authors failed to reach a firm conclusion concerning the pathway by which *L. monocytogenes* placed on the nasal mucous membrane or in the conjunctival sac arrived in the brain to produce encephalitis. Pallaske,[3] from both experimental results and from the fact that diseased sheep usually showed a purulent nasal exudate which sometimes contained *L. monocytogenes*, advocated that *L. monocytogenes* placed on the nasal mucous membrane reached the ethmoidal region and then passed to the brain via the olfactory nerve. However, this opinion contradicts the fact that the lesions in encephalitic listeriosis are found almost exclusively in the brain stem with no lesions at the olfactory bulb. Burn,[6] on the morphological aspects of the lesion, postulated that the bacterium entered the brain through the



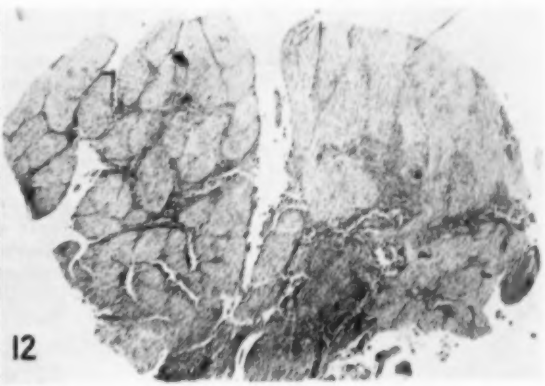


Fig. 11—Mononuclear round cell infiltration of the root of the trigeminal nerve in artificially induced encephalitic listeriosis in a goat. x 50.

Fig. 12—Mononuclear round cell infiltration in and between the nerve fibers in the root of the trigeminal nerve of a goat naturally infected with encephalitic listeriosis. x 20.

circulatory system. He injected *L. monocytogenes* into the blood vessels of both rabbits and monkeys, but none of the animals developed encephalitis. The only lesions seen were insignificant degenerative changes of the endothelial cells of the small blood vessels in the brain. Olson *et al.*[7] and Olson [8] suggested the possibility that a virus might act in collaboration with *L. monocytogenes*, but no positive evidence supporting this hypothesis has been found. Gill,[1] Biester and Schwarte,[14] and Naerlana [15] succeeded in producing encephalitis in sheep by intranasal, subcutaneous, and intravenous inoculations.

The present authors inoculated goats with *L. monocytogenes* by intranasal, subcutaneous, intraperitoneal, intravenous, intracarotid, and intracerebral inoculations but did not produce lesions of encephalitis as are found in naturally infected animals in which the lesions are distributed selectively in the brain stem.[10] Slight lesions were produced in the brain, but the distribution of these lesions was irregular without any tendency toward localization in the brain stem. In most instances, lesions also were found in the visceral organs. Intracarotid inoculation produced lesions in the meninges and ependyma of the cerebral ventricles and aqueduct, accompanied by lesions in the adjoining cerebral tissues and in the visceral organs, particularly the liver. Intracerebral inoculation produced lesions throughout the central nervous system and meninges without any changes in the other organs. In contrast, conjunctival instillation in a goat produced pathological changes similar to those observed in naturally infected animals even though they showed no signs of central nervous system disturbance. In this goat, the lesions were found in the pons and medulla oblongata. Moreover, the most characteristic finding was that the lesions were localized in the spinal tract of the trigeminal nerve and, furthermore, in the trigeminal nerve on the side where *L. monocytogenes* was inoculated, a fact suggesting invasion of the bacterium through the trigeminal nerve.

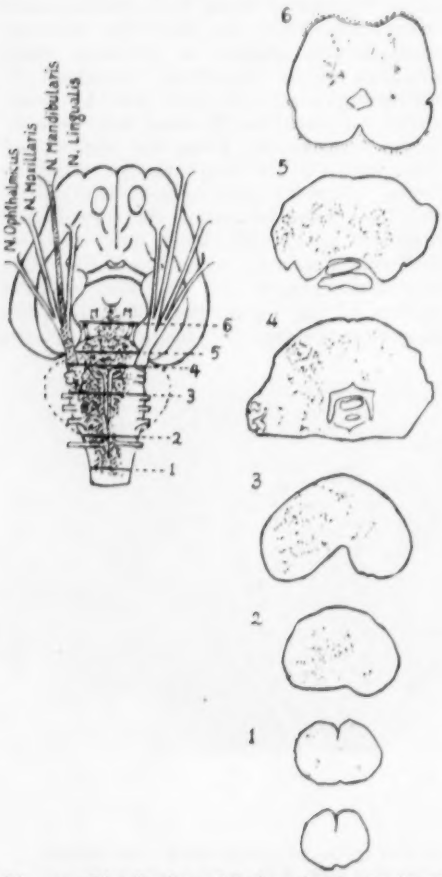


Fig. 13—Distribution of lesions in the brain of naturally infected goat 531.

Attempts also were made to produce encephalitis in mice, rabbits, and goats by conjunctival instillation, directed at the ophthalmic nerve, and by application of the organism to the oral and nasal mucous membranes after making minute wounds, directed at the maxillary, mandibular, and lingual nerves. Further studies imitating the probable method of natural infection were carried out by feeding Niboshi contaminated with *L. monocytogenes*. By this method, many of the inoculated animals developed encephalitic symptoms. Clinical findings on these animals were quite similar to those observed in naturally infected animals. Lesions in these animals were localized chiefly in the trigeminal nerve and *L. monocytogenes* was isolated from them. These findings suggest that *L. monocytogenes* spreads to the medulla oblongata by way of the trigeminal nerve. In support of this, histological studies on naturally infected sheep and goats revealed selective

distribution of the lesions in the trigeminal nerve and its branches.

SUMMARY

A goat inoculated with *Listeria monocytogenes* by conjunctival instillation, although not showing signs of infection, developed lesions along the trigeminal nerve, from the eye to the brain stem. Similar lesions were produced in rabbits by conjunctival instillation and inoculation through minute wounds in the lip, even though the animals remained asymptomatic. Encephalitic listeriosis was produced in mice either by applying the organism on minute wounds in the oral or nasal mucous membranes or by feeding small fish dried after boiling (Niboshi) soaked with a suspension of *L. monocytogenes*. The same methods of inoculation were used successfully to produce typical encephalitic listeriosis in goats. The lesions found in artificially infected animals were essentially the same in character and distribution as those observed in naturally infected animals. These results suggest that, in naturally occurring encephalitic listeriosis, *L. monocytogenes* invades the oral or nasal mucous membranes and travels along the trigeminal nerve to the brain stem.

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Preliminary Studies on Some Effects of a New Thiobarbiturate in the Dog

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THE STUDY described here was undertaken to evaluate some of the anesthetic properties of a new thiobarbiturate in the dog. The drug under investigation is the sodium salt of methyl-thio-ethyl-2-pentyl-thio-barbituric acid and has tentatively been designated as Sch 3132. Like thiopental and other thiobarbiturates, this compound is an ultrashort-acting barbiturate. Laboratory studies indicate that its anesthetic potency in the dog is approximately two thirds that of thiopental and that both drugs produce an equivalent amount of respiratory depression. It is somewhat more likely to produce laryngospasm and salivation.[1] Some reports have appeared in the European literature on the clinical use of the compound in small animals.[2,3]

MATERIALS AND METHODS

A total of 54 experiments were performed on 29 different dogs. The experiments may be grouped as follows:

Group No.	No. of experiments	Dose of thiobarbiturate (mg./kg.)	Rate of injection (seconds)
1	5	22.5	20
2	5	30.0	20
3	5	45.0	20
4	20	45.0	40
5	4	45.0	40
6	8	45.0	90
7	2	50.0	90
8	5	60.0	40

The animals in group 5 received atropine sulfate in a dose of 0.6 mg. (0.01 gr.), intramuscularly, one-half hour prior to administration of the thiobarbiturate.

In all instances, the thiobarbiturate was injected intravenously in a 5 per cent solution. This was prepared immediately before use.

The following were observed in each experiment: pulse rate, breathing rate, reflex activity, degree of salivation and muscular relaxation, and the character of the induction and recovery periods of anesthesia. The reflexes employed in evaluating the level of anesthesia were the palpebral, pad-pinch, and masseter muscle tone. The latter consisted of determining the degree of resistance in opening the jaws.

The rate of injection of the anesthetic was timed carefully. At least one control group of recordings were made prior to injection of the drug. Observations were made one minute following injection and thereafter at five-minute intervals during anesthesia until the animal was able to raise its head or roll up on its sternum.

RESULTS

Dosage and Rate of Injection.—The dosage recommended by the manufacturer of Sch 3132 was 45 mg. per kilogram of body weight and, therefore, this was employed in the largest group of experiments (group 4). With this dose given within a period of 40 seconds, a satisfactory level of surgical anesthesia was achieved, although 1 animal died shortly after injection. The rate of injection appears to influence the activity of the drug. When 45 mg./kg. was given in 90 seconds, respiratory depression was increased and cyanosis appeared in three of the eight experiments. With doses of 50 or 60 mg./kg., these effects were also observed and 2 of 7 animals died. Doses lower than 45 mg./kg. (*i.e.*, 22.5 or 30.0 mg./kg.) did not produce levels of surgical anesthesia.

Induction and Recovery.—In general, induction and recovery from anesthesia were smooth and rapid. There was no obvious excitement during injection of Sch 3132 and, within one minute after administration of a suitable dose, the dogs were unconscious. With doses which produced levels of surgical anesthesia (45 mg./kg.), the average time from injection of the drug until the animal was able to raise its head was 48 minutes, with a range of 16 to 91 minutes (fig. 1). The dogs were able to stand in an average of 68 minutes after induction of anesthesia (range 33 to 134 minutes). The longer recovery periods

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This investigation was supported in part by Schering Corp., Bloomfield, N. J. The thiobarbiturate under study has been designated Sch 3132 and is also known as neraval sodium and, in Europe, under the trade name of thiogenal.

were usually associated with the larger doses but some individual animals showed considerable variation with the same doses. Only 1 dog showed excitement during recovery, although all animals exhibited some degree of ataxia for a short period of time after getting up.

Breathing Rate (Fig. 2).—In all experiments in which a dose of 45 mg./kg. or more was employed, a marked reduction in breathing rate occurred within five minutes after injection of the thiobarbiturate. In general, the average rate was reduced to approximately one half of the control value and this lowered rate persisted for up to 45 minutes. In four experiments, respiratory paralysis and death occurred within five minutes after injection of the drug (*see* under "Mortality").

Pulse Rate (Fig. 2).—Tachycardia was a constant feature of the action of Sch 3132. This occurred within five minutes after injection of the drug and was most marked when doses of 45 mg./kg. or larger were used. The increased rate persisted for approximately 30 minutes, although in the experiments in which 45 mg./kg. was given in 90 seconds, or in those in which 60 mg./kg. was employed, it persisted for longer periods of time.

Reflex Activity.—The palpebral reflex was never completely absent in any of the experiments. In approximately 50 per cent of the experiments in which doses of 45 mg./kg. or larger were employed, the masseter muscle tone was observed to be absent at some stage of anesthesia. However, this reflex is difficult to evaluate and was not considered to be an accurate guide to anesthetic depth. A more precise measurement of anesthetic depth and duration was the pad-pinch reflex (fig. 1). This reflex disappeared in all dogs receiving doses of 50 or 60 mg./kg. and in those in which 45 mg./kg. was given in 90 seconds. In the experiments in which 45 mg./kg. was given in 20 or 40 seconds, the reflex disappeared in approximately 50 per cent of the dogs. In dogs receiving lower doses (22.5 or 30.0 mg./kg.), the reflex was not abolished in any instance.

Salivation (Fig. 3).—Excessive salivation occurred in 66 per cent of the experiments. This was most marked when lower doses (22.5 or 30.0 mg./kg.) were employed. Some dogs in these groups secreted as much as 20 to 30 ml. of saliva. The 4 dogs which received atropine sulfate prior to anesthesia were those, which in earlier experiments with Sch 3132 alone, showed profuse salivation. The use of atropine resulted in a marked reduction in the

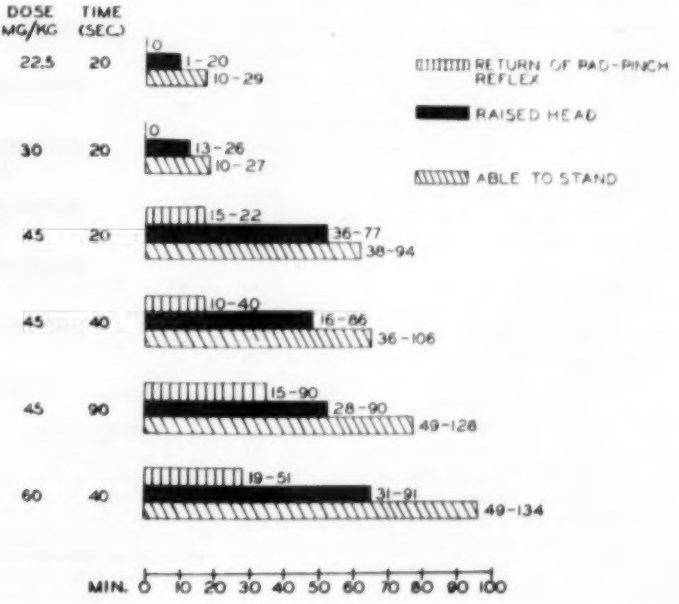


Fig. 1—The effect of various doses and rates of injection of Sch 3132 on the duration of anesthesia. The bars indicate average times; the figures at the end of the bars indicate ranges.

amount of saliva produced although in 2 of the 4 dogs some salivation occurred.

Voluntary Muscle Activity (Fig. 3).—Excessive voluntary muscle activity was observed in 64 per cent of the experiments.

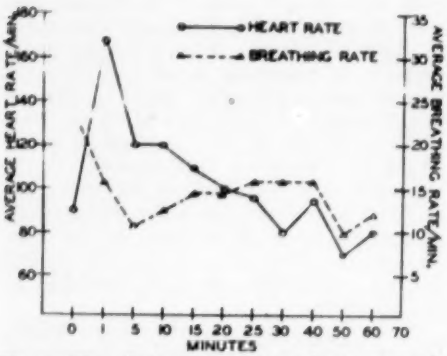


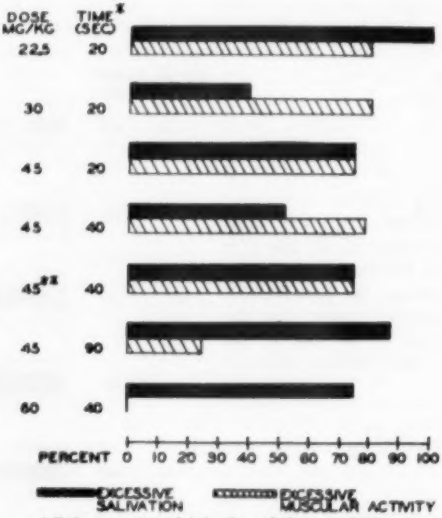
Fig. 2—The effect of Sch 3132 on heart rate and breathing rate. Note the marked fall in breathing rate and the increase in heart rate which occurs within one minute after injection of the anesthetic. In these trials, a dose of 45 mg. per kilogram of body weight was injected within 40 seconds.

"Champing" of the jaws was a common observation during induction of anesthesia and was particularly violent in dogs receiving the lower doses of Sch 3132. The champing simulated the so-called "chewing gum fits" of distemper encephalitis in dogs and was characterized by marked salivation. In a number of experiments, there was some degree of clonic spasms of one or more of the extremities. In many instances, the spasms were unilateral and resembled those seen in rhythmic myoclonia of distemper. This form of activity was most noticeable during the recovery period.

Mortality.—Four animals died within five minutes after injection of Sch 3132. One death occurred following the accidental injection of the thiobarbiturate at a rapid rate and, therefore, this animal was not included in computing the anesthetic mortality. The other 3 dogs received doses of 45, 50, and 60 mg./kg. given at the rate of 40, 90, and 40 seconds, respectively. All of these animals appeared to be in good health and would undoubtedly have been considered as suitable subjects for anesthesia. The gross necropsy and histopathology of these animals revealed only visceral congestion. There were no pathological changes which could be directly related to the action of the anesthetic. Thus, there were three deaths in 53 trials, a mortality rate of 5.9 per cent. As no other anesthetic agent was used for comparison under similar conditions, and since fixed dosages and rates of injection were employed in these experiments, this figure may not represent the true mortality. Under clinical conditions, anesthetics of this type are given to effect and individual variations of patients are taken into consideration.

DISCUSSION

When the thiobarbiturate, Sch 3132, was administered in the recommended dose of 45 mg./kg., satisfactory levels of surgical anesthesia were obtained. Larger doses may result in undesirable degrees of respiratory depression and lower doses do not produce satisfactory anesthesia. The rate of injection appears to influence the activity of the drug; a dose of 45 mg./kg. given in 90 seconds resulted in more respiratory depression than the same dose injected in 40 seconds.



* Refers to rate of injection of the anesthetic.

** This group of dogs received atropine sulfate (0.6 mg.) one-half hour before administration of the anesthetic.

Fig. 3—The percentage of dogs showing excessive salivation or excessive muscular activity ("champing fits" or clonic spasms of the limbs) following various doses of Sch 3132.

In these experiments, the pad-pinch reflex offered the most satisfactory guide to the level and duration of anesthesia. In the 20 experiments in which a dose of 45 mg./kg. was given in 40 seconds, this reflex was abolished in 10 animals and did not reappear for an average of 20 minutes (range 10 to 30 minutes) after injection of the drug. In the remaining experiments, the reflex was reduced but did not completely disappear. In this same dose group, the dogs were able to raise their heads in an average of 43 minutes (range 16 to 86 minutes) and were able to stand in an average of 62 minutes (range 38 to 106 minutes) after injection of the anesthetic. Induction and recovery were quiet in all animals.

Like other barbiturates used as anesthetics for dogs, Sch 3132 produces a reduced respiratory rate and an increased pulse rate. Two undesirable features were the excessive salivation and the increased voluntary muscle activity. In the group of dogs receiving 45 mg./kg. in 40 seconds, excessive salivation occurred in 50 per cent and excessive muscular activity in 75 per cent.

Variations in effect were observed in dogs receiving the same doses. For example, in seven instances in which 45 mg./kg. was given in 40 seconds, the animals were able to walk in 60 minutes or less, whereas in three instances they were unable to get up for 90 minutes or longer.

One dog died after receiving 45 mg./kg. injected in 40 seconds. In this animal, and in 2 other dogs which died with larger doses, respiratory failure appeared to be the primary cause of death; the heart continued to beat for a short time following cessation of breathing.

SUMMARY

The anesthetic properties of a new thiobarbiturate, designated as Sch 3132, were studied in dogs.

Doses of 45 mg./kg. of body weight given intravenously in 40 seconds produced light to moderate levels of surgical anesthesia lasting for approximately 20 minutes. Induction and recovery from anesthesia was smooth and rapid. Larger doses of the drug may produce undesirable degrees of respiratory depression and associated cyanosis.

In three of 53 experiments (5.9%), dogs died within five minutes after injection of the drug. Since fixed dosages and rates of injection were employed, this may not represent the true mortality rate. The primary cause of death appeared to be respiratory paralysis.

Profuse salivation and excessive activity of voluntary muscles frequently occur during surgical anesthesia with Sch 3132. The excessive salivation can be controlled with atropine. These undesirable features may limit the clinical usefulness of this thiobarbiturate.

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Vesicular Stomatitis in Deer

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Prior to 1943, horses and cattle were the only known natural hosts of the virus of vesicular stomatitis. In that year, an outbreak of the disease occurred among swine in an anti-hog cholera serum plant.[1] Since 1952, vesicular stomatitis in swine and other animals has been recognized every year in certain areas of the southeastern states. This poses a problem to individuals concerned with the diagnosis and eradication of vesicular exanthema and foot-and-mouth disease.

In June, 1955, a cooperative project was set up between the U. S. Department of Agriculture and the Wisconsin Agricultural Experiment Station to study the epizootiology of vesicular stomatitis in Georgia and adjoining states. During that summer, a serological survey was conducted to obtain information relative to the numbers and species of animals involved. It was found that over 50 per cent of the horses, cattle, and swine tested in the region drained by the Altamaha River and its tributaries carried specific virus-neutralizing antibodies, indicating prior infection. Serum samples from raccoon and deer in the area showed a high proportion of positive serum-neutralization titers.[2] At the same time, numerous reports were received of deer being observed with mouth and foot lesions. Some of these animals were so debilitated that they were overtaken and killed by farmers' dogs. Several similarly affected deer had been shot by hunters and had been found unfit for food. As a result of these observations, a study was undertaken of experimentally induced vesicular stomatitis of deer.

Materials and Methods

Five male deer * were exposed to the virus of vesicular stomatitis. Since the natural means of exposure and the relative susceptibility of the species were unknown, several different routes of inoculation were used initially to ensure infection.

The strains of virus were isolated from swine in Georgia and Louisiana during the summer of 1955. These isolates were examined by means of the complement-fixation test at the U.S.D.A. Animal Disease and Parasite Research Branch laboratory at Beltsville, Md., and found to be NJ-type vesicular stomatitis virus. Vesicular tissue was then submitted to the Wisconsin station where the virus strains were propagated in embryonating eggs, mice, and swine. Allantoic fluids from the first and second passages in eggs were used for the deer inoculations. These fluids had a titer of 10^6 l.d.$_{50}$ in chicken embryos.

A portable cattle-holding chute was used for restraint. Inoculated deer were kept in this for the duration of the infection. In this way, animals were confined sufficiently to permit the necessary handling and close observation, yet recumbency and limited forward and backward walking was allowed. A diet of a good quality of alfalfa hay, ground mixed grain dairy concentrate, and water was supplied at the anterior end of the chute.

Inoculations were made with the animals under barbiturate narcosis. For this purpose, pentothal sodium, pentobarbital sodium, or secobarbital sodium were used. These drugs were given intravenously to effect.

Preinoculation blood samples were taken for serum-neutralization tests. Body temperatures were recorded where possible. Three of the deer were found to be too excitable to permit readings to be taken without applying undue restraint. Virus titrations were carried out on 3 of the animals to obtain information on the relative susceptibility of deer to vesicular stomatitis. Virus was inoculated by intradermal and intramucosal routes; 0.1 ml. of inoculum was introduced at each site. Six serial tenfold dilutions of allantoic fluid were inoculated at different sites on the tongue as a method of virus titration. Similar dilutions were placed intradermally in the coronary bands or interdigital skin of the feet.

Two of the animals (D53, D54) were destroyed

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* Whitetail deer (*Odocoileus virginianus*) raised in Wisconsin.

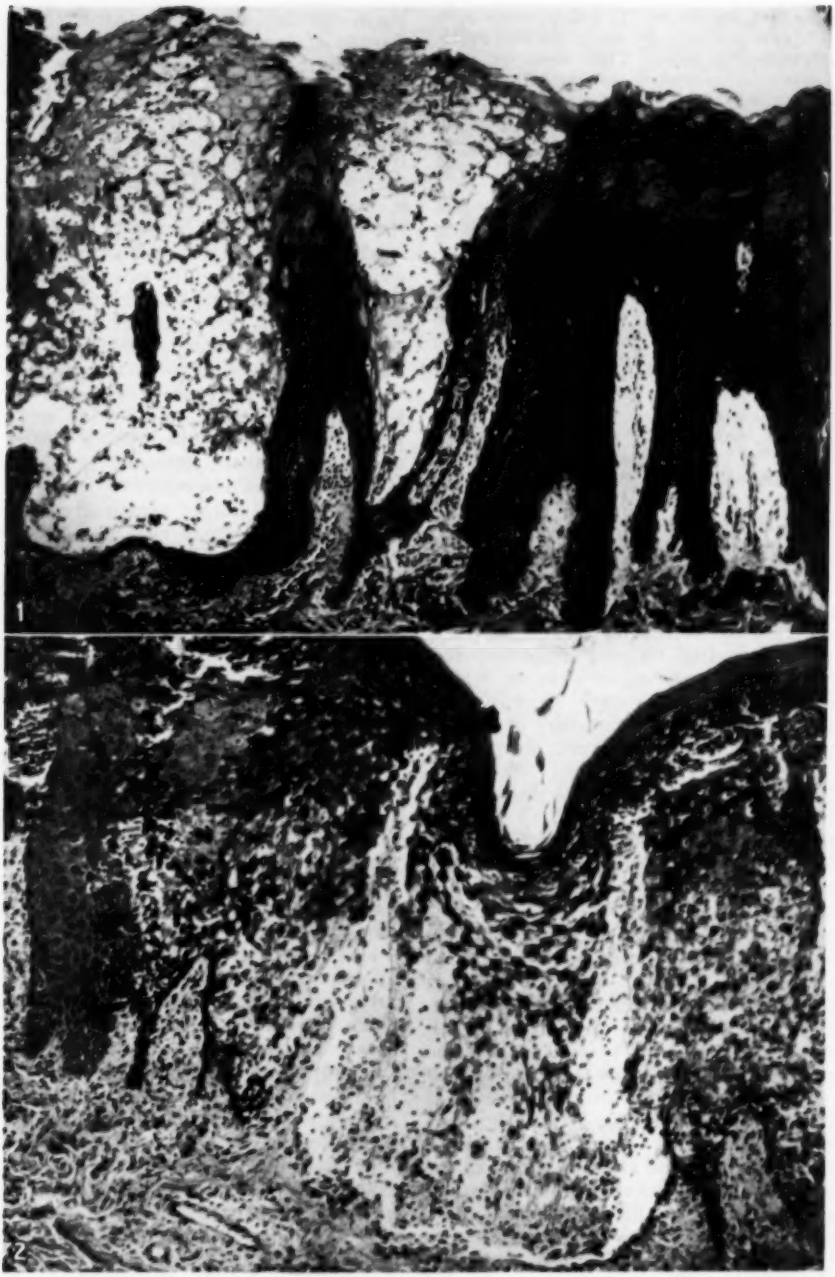

Fig. 1—Vesicle from tongue of deer D54 taken 50 hours postinoculation. Hematoxylin and eosin stain; x 240.

Fig. 2—Vesicle from muzzle of deer D54 taken 50 hours postinoculation. Hematoxylin and eosin stain; x 240.

at the peak of the disease (72 and 50 hours postinoculation, respectively) to study the histopathological changes produced. Three deer were held sufficiently long to observe specific antibody response by measurement of convalescent serum-neutralization titers. Preinoculation and postinoculation serum samples were examined for the presence of specific virus-neutralizing antibodies by inoculation of quantities of heat-inactivated serum mixed with equal amounts of selected virus dilutions into 9- or 10-day embryonating eggs. Serum-neutralization titers were expressed in chicken embryo $l.d._{50}$ units.

Recovery of virus was attempted from the blood, lungs, spleens, livers, lymph nodes, kidneys, and tongues of the 2 destroyed animals. The method of recovery used was inoculation of suspensions of ground tissues into 8- to 10-day embryonating eggs.

Euthanasia of animals at the end of the experiment was accomplished by intraperitoneal injection of one of the barbiturates or a solution of chloral hydrate.

A list of animals used and methods of exposure are given (table 1).

RESULTS

All preinoculation serum samples were negative when examined for the presence of neutralizing antibodies against NJ-type vesicular stomatitis virus. Febrile response was detected in the 2 animals on which adequate body temperature records were obtained. The body temperature of animal D51 rose from an apparent normal of 101.3 to 101.8 F., to a peak of 104.3 F. at 24 hours postinoculation. The decline following this was at first rapid to 102.6 F. at 40 hours, then more gradual to normal again at 80 hours after inoculation. The other deer, D55, showed an elevation of temperature to 103.0 F. at 24 hours after inoculation followed by a rapid return to normal. Vesiculation was not observed in this animal.

Three of the 4 animals exposed by intramucosal inoculation of the tongue developed tongue vesicles. These took the form of small circumscribed areas at the sites of inoculation, appearing first at 24 to 48 hours postinoculation (fig. 1). Little vesicular fluid developed in the affected areas. The surface of such areas was not raised above the level of the surrounding normal epithelium but, on histological examination, true vesicles were evident. Because the early stages of vesiculation were not available for examination (only at 50 and 72 hours postinoculation), the true picture of vesicular development could not be followed exactly. The vesicles arose from confluence of lacunae which formed between the cells and from final cellular disintegration. The prickle cell layer apparently was first affected. Evidently, the cells in this area shrank and became rounded. The intercellular bridges were disrupted and destroyed. Eventually, many of the epithelial cells completely disintegrated and the lacunae became confluent to form distinct vesicles of significant size. Epithelial cell destruction usually extended down to the basal layer and sometimes even this was destroyed over small areas. Moderate numbers of polymorphonuclear leukocytes and macrophages invaded the vesicles but very few of these cells were present in the subepithelial tissues. Congestion and even hemorrhage of the subepithelial tissues was a constant feature immediately under the vesicles but not elsewhere.

Surface epithelium remained intact for a relatively long time (fig. 2) as compared with observed vesicle development in cattle and swine, where fluid pressure inside the vesicle caused bulging of the surface followed by early rupture of the vesicle.[3] Surface epithelium sometimes seemed to remain intact until cellular degeneration in deeper layers became complete. Then sloughing sometimes occurred without the vesicle ever having ruptured.

Gross foot lesions were not observed. A single microscopic lesion, vesicular in nature, was observed in routine histological examination of inoculated interdigital skin from the animal which was killed 50 hours after inoculation. It was thought that possibly vesiculation in the more heavily skinned coronary or interdigital regions was a slower process which was being aborted by the rapid antibody response to tongue infection. Accordingly, an attempt was made to induce foot lesions in one animal (D55) by inoculation of coronary bands as the only route of exposure. This animal manifested a transient pyrexia following inoculation but without development of gross foot lesions. Histological examination was not made as the animal was allowed to recover for demonstration of antibody response.

Salivation was moderately increased in those animals which developed tongue vesicles. Affected animals lost weight due to their disinclination to eat when mouth lesions were present. At this time, alfalfa

hay was taken in preference to finely ground dairy concentrate. Histological sections made from lesions on the tongue of the deer killed 72 hours postinoculation showed small foreign bodies embedded in the degenerating epithelium (fig. 3). Application of the Hotchkiss-McMannus differential stain [4] indicated that these particles were of plant origin. Some of these foreign bodies were found penetrating the submucosa.

One of the deer (D52) appeared "refractory" in that visible lesions did not develop following inoculations into the tongue, muzzle, and feet. As the deer was an extremely nervous animal, his temperature was not taken. A high serum-neutralizing titer was found two weeks after exposure.

Virus was recovered only from vesicular tissues or fluids taken from inoculated animals during the early stages of the disease. Viremia may have existed but virus was not detectable in blood taken at 50 and 72 hours postinoculation. Lung, liver, spleen, lymph node, and kidney tissues gave negative results when examined for virus at 50 and 72 hours.

Virus titration by tongue inoculation was attempted in animals D52, D53, and D54. Titrations were read at 48 hours after inoculation. Titers of 10^6 ID per milliliter were obtained in animals D53 and D54, evidenced by the development of lesions at the sites of inoculation of 0.1-ml. quantities of allantoic fluid diluted to 10^{-5}. These allantoic fluids titrated 10^5 $l.d._{50}$ units of virus per milliliter in 8-day chicken embryos. Material from the same strain (Adamson), a different harvest, but one which also had an embryo titer of 10^5 $l.d._{50}$ was titrated on the tongue of a mature cow and in the coronary band of a 40-lb. pig.

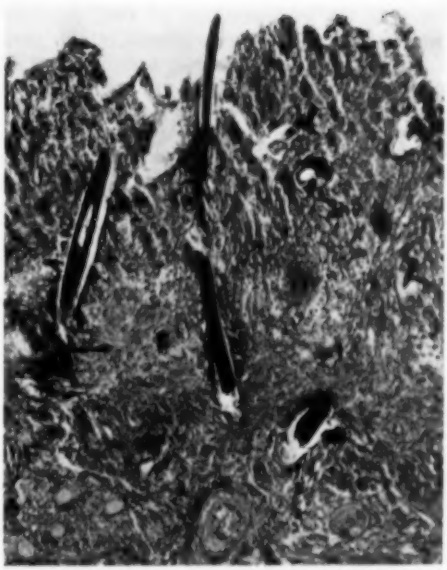

Fig. 3—Vesicle from tongue of deer taken 72 hours postinoculation. Plant particles are the strawlike dark-staining bodies. Stained by Hotchkiss-McMannus method; x 160.

The titer in the cow was 10^2 and in the pig 10^4. Deer D52 gave no clinical evidence of infection.

Serums of the 3 deer which were held sufficiently long to allow observation of virus-neutralizing antibody response neutralized 10,000 $l.d._{50}$ of NJ-type vesicular stomatitis virus when examined 14 days after exposure.

The results of these experimental infections are summarized (table 1).

DISCUSSION

The observations made in this study enable us to better understand the role of

TABLE 1—Summary of Experimental Vesicular Stomatitis Virus Infections of Deer

Animal No.	Age	Sex	Route of exposure: Intramucosal inoculation	Route of exposure: Intradermal inoculation	Gross lesions	Febrile response	Convalescent SN antibody titer	Disposition
D51	8 mo.	M	Tongue	Muzzle, caudal folds, coronary bands	Vesicles on tongue and muzzle	++	10^4 $l.d._{50}$	Held for further experiments
D52	2 yr.	M	Tongue	Muzzle, interdigital skin	None	None	10^4 $l.d._{50}$	Destroyed 2 wk. postinoculation
D53	8 mo.	M	Tongue	Interdigital skin	Vesicles on tongue	Not determined	Not determined	Destroyed 72 hr. postinoculation
D54	2 yr.	M	Tongue	Coronary bands	Vesicles on tongue	Not determined	Not determined	Destroyed 50 hr. postinoculation
D55	1 yr.	M	None	Coronary bands	None	+	10^4 $l.d._{50}$	Held for further experiments

deer in the epizootiology of vesicular stomatitis. Finding the serums of 60 per cent of the deer in the enzootic area positive to the serum-neutralization test would lead one to suspect that deer are highly susceptible to vesicular stomatitis and that the disease produced is not fatal. This was found to be the situation. Our experimental findings help to explain the reports received from residents of the area that deer are seen with sore mouths and feet. Our failure to produce gross foot lesions does not prove that such lesions do not occur in natural infections. It is possible that environmental stress is an important factor in causing these changes. Foot involvement in cattle has been difficult to produce experimentally, yet it is seen in field cases of vesicular stomatitis.[5]

On the basis of limited observation, it may be said that clinically inapparent infections occur. These may be even more common under conditions of natural exposure. In such cases, animals would subsequently carry virus-neutralizing antibodies without having experienced clinical disease. At the opposite extreme, it is possible that the superimposition of environmental stresses could precipitate the development of severe lesions. It is readily seen how a grazing and browsing animal like the deer might be fatally incapacitated by severe mouth or foot involvement. The observed invasion of injured tissues by sharp plant particles may be a complicating factor in natural infections contributing to secondary bacterial infection. Therefore, one may suppose that, while an uncomplicated case of vesicular stomatitis in a deer may be mild or even inapparent, secondary stresses could greatly increase the severity of the attack.

It is unlikely that deer are reservoir hosts of vesicular stomatitis, since the experimental disease was short-lived and quickly followed by high levels of virus-neutralizing antibodies. However, deer may aid in maintaining the disease in its present enzootic proportions by supplying a generation of new susceptible hosts each outbreak season.

SUMMARY

1) Vesicular stomatitis in swine was recognized as an enzootic disease in southeastern United States in 1952.

2) Detection of neutralizing antibodies in deer from southern Georgia and reports of disease by hunters led to the consideration of deer as a possible reservoir of vesicular stomatitis.

3) Five deer were inoculated by several routes with vesicular stomatitis virus and developed mild, acute, or abortive infections.

4) Deer are probably not important reservoirs of the virus, since recovery is rapid. They may have a minor role in maintaining the summer epizootic. The disease in deer, although mild under experimental conditions, may be more serious under certain stress conditions existing in the field.

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A Polyvalent Toxoid for Botulism in Mink

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SINCE the first report by Hall and Stiles in 1938 [1] of botulism in mink, fur farmers have become interested in procedures for prevention of this disease. Although botulism in mink is uncommon, the mortalities in individual outbreaks have been high. In our experience, polyvalent antitoxin has been inadequate for control of the disease in some instances and it was evident that there was need for a safe, effective immunization procedure. Successful immunization of mink against *Clostridium botulinum* type C by Swedish workers prompted us to study the effects of immunization with combined toxoids.

REVIEW OF THE LITERATURE

Clostridium botulinum type C has been incriminated most often as the cause of botulism in this country, as well as Sweden.[1-5] Quortrup and Gorham [6] found that mink were susceptible to *Cl. botulinum* toxin types A, B, and C but were most susceptible to type C. Dinter and Kull [7] found mink to be 400 times more susceptible to type C than to type A toxin when given orally, but only five times more susceptible to type C when given parenterally.

Dinter and Kull [8] in 1950, in Sweden, developed an aluminum hydroxide-adsorbed type C toxoid. This toxoid proved to be safe and efficient when used to protect white mice and guinea pigs against the type C toxin. It also protected mink against massive challenge that killed control mink within 12 hours. Dinter [9] found that guinea pigs vaccinated with the toxoid developed high serum antitoxin levels in five to seven weeks and that the titers remained high for at least eight months. In 1952, Kull and Moberg [10] reported on the vaccination of 45,000 mink on 60 farms. They used 1 ml. of *Cl. botulinum* type C aluminum hydroxide-adsorbed toxoid. Botulism occurred on one of these farms where 3,920 mink had been vaccinated and 30 left unvaccinated with 40 fatalities, 10 from the vaccinated group and all 30 of the control animals.

Kull [11] later determined that his botulinus C toxoid gave good protection for at least three and one-half years.

Larsen *et al.*,[4] in 1955, also described the successful immunization of mink against *Cl. botulinum* toxin type C by using potassium alum-precipitated toxoid. The duration of immunity in their test was at least 52 days.

The most practical immunization program in mink would entail the use of combined A, B, and C toxoids—all the types demonstrated to cause intoxication in mink. The present report describes the preparation of polyvalent toxoids and the immunization of mink with this preparation.

MATERIALS AND METHODS

Toxins.—The toxins used for the preparation of toxoid and for challenge of animals were obtained from bacteria that had been cultivated in cellophane® bags. The technique differed in design, but not in principle, from that described by Sterne and Wentzel.[12] The apparatus used for the cultures is of simple construction and is easily prepared for use (fig. 1). After filling the cellophane bag with 0.85 per cent saline solution and dispensing the medium into the cylinder, the unit was assembled and autoclaved. When the temperature of the unit had dropped to approximately 37 C., the inoculum was introduced into the bag and the unit was incubated at 37 C. for several days. In some early experiments, actively growing dialysis-bag cultures were used as inoculums but, later, the use of washed spores was found to be simpler. The exact period necessary for maximum toxin production varies with the type of organism and the kind of medium used. Subsidence of gross activity within the bag was taken as a criterion of the maturity of the culture and the climax of toxin production. When it was desired to remove the culture without further treatment or to sample it, glass tubing was inserted through the top and into the glass U-tube. Alternately, the entire bag and lid assembly was removed, the bag dipped into distilled water to rinse it and pierced at the bottom to drain the entire culture. If the whole culture was to be used for toxoid, the entire assembly was transferred to another jar containing buffered formalin (*see* below). Approximately 600 ml. of highly potent toxin was obtained from each bag. The sources of the inoculums, the mediums used, and the titers of toxin obtained were as follows:

Type A.—The inoculum for this culture and for the type B culture and also the formulas for the mediums for those cultures were obtained from Duff.[13] A tube of cooked meat medium was in-

From the U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Mont. (Bell); Agricultural Experiment Station, State College of Washington, Pullman (Farrell); and Fur Animal Disease Research Laboratory, U. S. Department of Agriculture, Pullman, Wash. (Gorham).

oculated with the spore suspension and from this culture a dialysis-bag culture was initiated in a small vessel. About 10 ml. of actively growing culture were used to inoculate each of the two large bags.

The medium was composed of the following ingredients: casitone (Difco), 2.0 per cent; yeast extract (Albimi), 0.5 per cent; glucose (added after sterilization), 0.5 per cent; and NaOH to make pH about 7.1 to 7.2 before sterilization.

The cultures were incubated for six days. At the end of that period, the approximate titer of toxin, determined by the intraperitoneal injection of serial tenfold dilutions into mice, was found to be 10^7 m.l.d. per milliliter.

Type B.—The spore suspension obtained was used to initiate a small culture which, when actively growing, was used to inoculate the large dialysis bags. The formula for type B medium was as follows: casitone (Difco), 1.5 per cent; yeast extract (Albimi), 0.5 per cent; cysteine HCl, 0.075 per cent; glucose (added after sterilization), 0.5 per cent; and NaOH to make pH about 7.1 before sterilization.

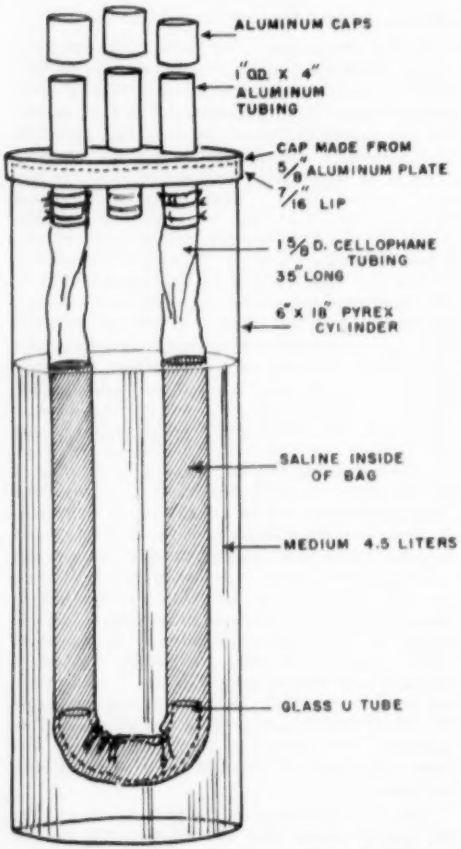
ALUMINUM CAPS
1" OD X 4" ALUMINUM TUBING
CAP MADE FROM 5/8" ALUMINUM PLATE
7/16" LIP
1 5/8 D. CELLOPHANE TUBING 35" LONG
6" X 18" PYREX CYLINDER
SALINE INSIDE OF BAG
MEDIUM 4.5 LITERS
GLASS U TUBE

Fig. 1—The apparatus used for cultivating the bacteria from which the polyvalent toxoid for botulism in mink was made.

The cultures were incubated for five days and titrated in mice by the injection of tenfold and twofold dilutions. The titer was 1×10^7 m.l.d. per milliliter.

Type C (1) Toxin for Toxoid Production.—The toxigenicity of *Cl. botulinum* type C is notoriously variable.[6] However, we have derived a strain which retains maximum toxigenicity by constant selection for that factor.[14] Washed spores of this strain were used to initiate a small dialysis-bag culture. This culture in turn was used as the inoculum. The medium used was a composite of two mediums which we have found useful, *viz.*, yeast autolysate-lactalysate medium suggested to us by Dr. Wayne I. Jensen and the corn steep medium of Sterne and Wentzel.[12] Either of the mediums produced good titers of toxin but, in the former, growth was too rapid with much foaming and, in the latter, growth was too slow.

We found that a mixture composed as follows was most satisfactory: lactalysate (Baltimore Biological Laboratory), 0.75 per cent; yeast autolysate, 0.5 per cent; N-Z case (Sheffield Chemical Co.), 1.4 per cent; corn steep liquor (alkali treated), 2.25 per cent; starch, 0.1 per cent; ammonium phosphate [$(NH_4)_2HPO_4$], 0.1 per cent; and glucose (added to prepared medium before autoclaving)—1.0 per cent; a final pH of approximately 7.2 is desirable.

The medium was essentially 1 part of yeast autolysate and lactalysate medium and 3 parts of corn steep medium with the addition of starch and ammonium phosphate.

The type C cultures were harvested after five days of incubation. The titer of this toxin was 10^6 m.l.d. per milliliter (twofold dilutions were not done).

Type C (2) Toxin for Challenge.—In these cultures, washed spores were used as the inoculum. Incubation of the cultures continued for seven days. The titer of the whole culture was 2×10^6 m.l.d. per milliliter. The titer of the supernatant was 4×10^5 per milliliter. Supernatant was used for the challenge, because titration of clear solutions gave more reproducible results than titrations of toxin in whole cultures or resuspended sediments. The fact that much of the toxin in cultures may be within the bacterial cells as stated by Dozier,[15] in 1924, is now generally recognized.[16]

Toxicity of the supernatant toxin for mink by the enteral route was determined by giving 5-ml. volumes of several dilutions to mink kits (390 to 540 Gm.) by gavage (table 1). The toxin was administered through a tube made from a bovine artificial insemination tube connected to a 10-ml. syringe by a small section of latex tubing. After the initial determination of toxicity, the toxin was stored for 129 days at −20 C. and tested again, this time in larger animals (950 to 1,200 Gm.).

No evidence of difference in susceptibility between young and old mink or loss of toxicity was demonstrated; however, there may have been smaller differences

TABLE 1—Toxicity of Supernatant Toxin for Mink by the Enteral Route

No. of doses, mouse $l.d._{50}$	Toxicity for mink (No. dead/total challenged)	
	Before storage	After storage
1×10^5	5/5	5/5
1×10^4	0/5	0/5
1×10^3	1/5	1/5
1×10^2	0/5	0/5

than the differences (tenfold) in dosages. Deaths of 2 animals that received relatively small doses of toxin (10^3 m.l.d.) can probably be accounted for on the basis of wide variations in susceptibility. Similar variations were frequently demonstrated in the enteral titration of toxin in guinea pigs and fowl. For the purpose of determining a challenge dose, it was considered, somewhat arbitrarily, that the $l.d._{50}$ dose was approximately one half of the difference between the largest and second largest doses. Multiples of 55,000 (5.5×10^4) mouse m.l.d. were used for challenge.

Detoxification in Formalin.—The procedure for detoxification of toxin differed somewhat from the usual method in that the formalin was not added directly to the the toxin but was permitted to dialyze into it. The desired amount of filtrate, supernatant, or whole culture, usually 200.0 to 500.0 ml., was put into a dialysis bag. The bag was then suspended in 5 liters of M/150 phosphate buffer at pH 5.8[12] containing 20.0 ml. of reagent grade formalin (assay 36.3 % formaldehyde). The exact percentage of formalin was unimportant inasmuch as the rate of detoxification was determined by actual test, but we found that the concentration given above was satisfactory. It was not desirable to use very concentrated formalin inasmuch as detoxification proceeded rapidly and excessive exposure of toxoid to formaldehyde appeared to reduce antigenicity.[17] The entire assembly was placed in a 37-C. incubator and samples were taken at intervals of one, two, or three days for titration in mice. When 0.1 ml. of undiluted material failed to kill mice in four days, 0.5 ml. was tested and, if death did not occur in four days, the bag was removed from the formalin and immersed in 0.85 per cent saline at 5 C. to remove excess formalin and to continue the removal of dialysable metabolites and medium components. After a day or two, the toxoid was considered ready for alum precipitation.

Alum Precipitation.—Alum precipitates of the three toxoids were prepared according to the method of Rice *et al.*[18] In the case of the A toxoid, a filtrate was used (sintered glass, UF porosity). Supernatant was taken for type C and whole culture for type B. The mixtures were placed in a cold room (5 C.) overnight. They were then centrifuged, the supernatant was decanted and the sediment was brought to the original volume (100.0 ml.) with 0.85 per cent saline.

All of the toxoids were prepared as experimental lots to test the method of preparation. Two of the lots (B and C) had not been completely detoxified. One tenth of a milliliter of each caused delayed death when injected into mice, and 0.25 ml. caused delayed death of guinea pigs. Since time did not permit the production of new lots and since it was thought that the minimal toxicity might not affect mink, a polyvalent toxoid was prepared by mixing 50-ml. volumes of each of the three toxoids. Ten mink kits, weighing from 390 to 520 Gm., were each given 2.0 ml. of the polyvalent toxoid and they showed no ill-effects.

Immunization.—On July 19, 154 mink kits were given 1.0 ml. of polyvalent ABC toxoid subcutaneously. Three days later, 1 female died. Necropsy and bacteriological examination did not reveal the cause of death but, possibly, the toxoid was responsible. The remaining 153 mink showed no untoward reaction.

Challenge.—Eighty-eight of the vaccinated mink kits were placed in four groups of 20 mink each and one group of 8 mink. The results of challenge with various doses of toxin 129 days after vaccination are shown (table 2).

DISCUSSION

These trials demonstrate that polyvalent ABC toxoid is safe and effective for mink when used under the conditions of these trials. Most of the mink immunized developed a high degree of resistance to type C *Cl. botulinum* toxin. The degree of resistance apparently varies widely in individual animals.

From the work done on immunization of mink against type C botulism, and especially that done by Dinter, Kull, and Moberg in the last few years, it is concluded

TABLE 2—Various Challenge Doses of Type C Botulinus Toxin Administered to Immunized Mink

Group No.	Dose (mouse m.l.d.)	Mortality	Remarks
I	2.75×10^5	1/20	Typical botulism in 1 mink
II	5.50×10^5	0/20	.....
III	8.25×10^5	0/20	.....
IV	1.10×10^6	2/20	Typical botulism in 2 minks
V	2.0×10^6	0/8	.....

that the field use of botulinus toxoid type C is an efficient procedure. Rice [10] adduced evidence that mixing of toxoids does not elicit diminished response to the individual components of the mixture, but that in fact an adjuvant effect may be demonstrated. Therefore, a single injection of polyvalent toxoid is a practical procedure. The dose of toxoid given to the mink in this experiment was probably excessive in view of the fact that Sterne and Wentzel [12] gave proportionately much less toxoid to cattle. Since we gave a dose of 1.0 ml. of equal parts of A, B, and C toxoids, or 0.333 ml. of each, it is evident that approximately 1,800 doses of one type of toxoid can be prepared from one culture jar.

The use of toxoid on mink farms in the United States awaits the time when such a product can be produced commercially at a cost that would be economically feasible for the mink rancher. Whether A and B toxoids should be included in an immunization program must await further investigations of field epizootics and trials as to the efficiency of such toxoids.

SUMMARY

A method is described for the preparation of toxoids of *Clostridium botulinum* types A, B, and C. The entire procedure from inoculation of culture to removal of excess formalin from the toxoid may take place in a closed system. One injection of a mixture of alum-precipitated toxoids thus produced was efficacious in protecting mink against large doses of type C toxin, the most common cause of botulism on mink farms.

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Investigations on the Endocrine-Controlled Defense Mechanism of Estrous and Pseudopregnant Rabbit Uteri

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THE UTERI of luteal phase rabbits [2,3] and cows [4] contain much greater numbers of live bacteria at a standard time after experimental inoculation than do the uteri of estrous phase animals. Inoculation of pseudopregnant rabbit uteri with *Escherichia coli* results in an accumulation of pus in the lumen and infiltration of the endometrium by large numbers of leukocytes. Despite this leukocytic response, large numbers of live *Esch. coli* can be recovered from the uterine lumens 24 to 28 hours after inoculation. In contrast to this, inoculated uteri of estrous rabbits show little gross response at the end of the same time interval and only moderately increased leukocytic infiltration of the endometrium, yet relatively few bacteria can be recovered.

This differential response to infection is due to the action of progesterone during the luteal phase [1,4] and is apparently restricted to the uterus.[3] Microscopic examination of debris from the previously inoculated uterine horns of several estrous and pseudopregnant rabbits has shown that leukocytes in both types of uteri often contain large numbers of ingested bacteria. Despite the leukocytic activity, the secretions of pseudopregnant rabbit uteri might constitute a more suitable medium for bacteria than secretions of estrous rabbits and thus cause a differential survival of organisms. Therefore, comparison was made of the luminal contents of estrous and pseudopregnant rabbit uteri with respect to their properties as bacterial culture mediums; this was repeated in sensitized uteri, *i.e.*, uteri that were first injected with dead *Esch. coli*.

MATERIALS, METHODS, AND RESULTS

The rabbits used in the experiment were domestic females, approximately 1 year old, which had been kept in individual cages for at least a month before use to minimize the chance of uncontrolled pseudopregnancy. Experimental pseudopregnancy was induced by mating estrous females to vasectomized bucks or by intravenous injection of 100 I.U. of chorionic gonadotrophin. Pseudopregnant rabbits were used eight, nine, or ten days after induction of ovulation.

Experiment 1.—Laparotomies were done consecutively on four replicates of 2 estrous and 2 pseudopregnant rabbits each. Each uterine horn was ligated at its cervix and tubouterine junction, and one horn was excised completely. Each horn was injected with 0.2 ml. of a suspension of *Esch. coli* and the excised horn was left in the peritoneal cavity with the intact horn. Four hours after inoculation, the rabbits were killed, both uterine horns were removed from the body cavity, and each horn was flushed as aseptically as possible with 5.0 ml. of sterile physiological saline. The washings from each uterine horn were plated serially on eosin methylene blue agar, incubated at 37 C. for 48 hours, and the colonies of *Esch. coli* counted. The total number of bacteria recovered from each uterine horn was then calculated.

The numbers of bacteria used to inoculate each uterine horn and the numbers recovered after four hours are shown (table 1). The mean logs of the number recovered from the four classes of uterine horns are as follows: intact estrous, 6.54; intact pseudopregnant, 8.3; excised estrous, 8.22; and excised pseudopregnant, 8.37. These logs correspond to numbers of 3,470,000, 200,000,000, 166,000,000, and 234,000,000, respectively. Statistical analysis showed that the difference between the two kinds of intact horns in total bacteria recovered was greater ($P<0.01$) than the difference between the excised horns.

This study suggested that the differential recovery of live bacteria from inoculated uteri of estrous and pseudopregnant rabbits is due to some factor other than a difference in the nutritive value of the

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TABLE 1—Log Numbers of Escherichia Coli Recovered from Intact and Excised Uterine Horns of Estrous and Pseudopregnant Rabbits

		Type of rabbit					
		Estrous			Pseudopregnant		
Replicate	Inoculum per horn	Rabbit No.	Intact horn	Excised horn	Rabbit No.	Intact horn	Excised horn
1	8.34	1	6.81	8.29	3	8.56	8.57
		2	6.63	8.27	4	8.72	8.59
2	8.59	5	5.76	8.04	7	8.45	8.18
		6	7.52	7.92	8	8.22	7.33
3	8.72	9	7.85	8.54	11	8.7	8.63
		10	6.43	8.52	12	7.54	8.66
4	8.38	13	4.02	7.88	15	7.66	8.45
		14	7.33	8.28	16	8.56	8.53

uterine secretions. A depressive effect of progesterone on the uterine defense mechanism of luteal phase animals was considered a possible explanation.

The difference between the two types of uteri is evident soon after inoculation; a distinct difference exists in the number of live *Esch. coli* which can be recovered from the intact uterine horns as soon as four hours after their introduction. This may be related to the observation that a moderate leukocytic infiltration of the rabbit endometrium is characteristic of estrus as contrasted to pseudopregnancy,[1] which suggests that the uterine defenses of the estrous animal are in a state of relatively greater readiness to combat a bacterial invasion than those of the luteal phase animal.

An attempt was next made to determine whether the defense mechanism of a pseudopregnant rabbit uterus could be made as effective as that of an estrous rabbit uterus by sensitizing (stimulating) the defense mechanism of the luteal phase rabbit with dead bacteria in the uterus prior to injection of live organisms.

Experiment 2.—(*a*) Two replicates of 2 estrous and 2 pseudopregnant rabbits each were used initially. Laparotomies were done on 1 estrous and 1 pseudopregnant rabbit of each replicate. The uterine horns were then ligated at each end, and 0.2 ml. of a heavy suspension of heat-killed *Esch. coli* was injected into each horn (to sensitize them) using a 27-gauge hypodermic needle. Fifteen hours later, the uterine horns of the 2 previously untreated rabbits were also ligated at each end and all 4 rabbits in the replicate were inoculated in each uterine horn with 0.2 ml. of a suspension of live *Esch. coli*. Four hours after inoculation, the rabbits were killed, their uteri removed, and each horn flushed as aseptically as possible with 5.0 ml. of sterile physiological saline. The washings from the two horns of each rabbit were pooled and aliquots were plated serially on eosin methylene blue agar and incubated at 37 C. for 48 hours and the colonies of *Esch. coli* were counted.

b) The next four replicates were comprised of 1 estrous and 1 pseudopregnant rabbit each; only one uterine horn of each rabbit was sensitized 15 hours prior to inoculation of both horns with live *Esch. coli*. These rabbits were also killed four hours after inoculation with live bacteria, each uterine horn was flushed with 5.0 ml. of saline, and an estimate was obtained of the number of live *Esch. coli* recovered from each horn.

The number of living *Esch. coli* used to inoculate each uterine horn and the numbers recovered after four hours are listed (table 2). The results of the first two rep-

TABLE 2—Log Numbers of Escherichia Coli Recovered from the Sensitized and Control Uterine Horns of Estrous and Pseudopregnant Rabbits

		Type of rabbit					
		Estrous			Pseudopregnant		
Replicate	Inoculum per horn	Rabbit No.	Sensitized horn	Control horn	Rabbit No.	Sensitized horn	Control horn
1	8.34	1, 2*	3.26	5.51	3, 4*	4.67	8.39
2	8.39	5, 6*	5.44	6.67	7, 8*	6.32	8.31
3	8.38	9	4.32	6.61	10	5.54	8.98
4	8.39	11	5.28	6.13	12	5.95	6.62
5	8.46	13	4.2	7.69	14	7.18	8.68
6	8.46	15	4.41	7.93	16	6.39	8.96

* Both uterine horns of each rabbit in replicates 1 and 2 were either sensitized or control. The log number in the table is that of one half of the actual number of bacteria in the inoculum or recovered from both horns, the log of the total number being in each case 0.3 greater than the log of one half the total number.

licates suggested strongly that the previous instillation of dead bacteria had a pronounced effect on the uterine defense mechanism of both estrous and pseudopregnant rabbits. The mean log of the actual numbers of live *Esch. coli* recovered from both horns of each type of uterus are as follows: sensitized estrous, 4.65; control estrous, 6.39; sensitized pseudopregnant, 5.8; and control pseudopregnant, 8.65.

Experiment 3.—In order to determine whether the apparent treatment effect on the uterine defense mechanism was a general uterine phenomenon or whether it was localized in the sensitized horn, dead *Esch. coli* were injected into only one uterine horn of the rabbits in the next four replicates.

The mean logs of the numbers of bacteria recovered from the four classes of uterine horns are: sensitized estrous, 4.55; control estrous, 7.09; sensitized pseudopregnant, 6.26; and control pseudopregnant, 8.31. The results of these replicates indicate that the injection of dead organisms prior to inoculation with live ones stimulated the defense mechanism of the sensitized uterine horn only.

Discussion

The data from all replicates were included in the statistical analysis since the treatment effect was apparently a strictly localized phenomenon. For purposes of obtaining over-all means, the log of one half of the actual number of bacteria recovered from both uterine horns of each rabbit in the first two replicates was used as an estimate of the number recovered from one horn. The mean logs of the numbers of bacteria recovered from the four types of uterine horns over all six replicates are as follows: sensitized estrous, 4.48; control estrous, 6.76; sensitized pseudopregnant, 6.01; and control pseudopregnant, 8.32. These logs correspond to numbers of approximately 30,200, 5,750,000, 1,020,000, and 209,000,000, respectively. Statistical analysis by the "t" test of the within-replicate log differences revealed significant differences ($P<0.01$ or $P<0.02$) between any two of the four groups.

The introduction of dead bacteria into the uteri of rabbits in either the luteal or follicular phase prepares the defense mechanism for greater effectiveness against live bacteria injected later. The uterine defenses of sensitized horns in pseudopregnant rabbits are more effective than those of control horns in estrous rabbits, even though estrous rabbit control horns have far superior defensive ability against *Esch. coli* than do pseudopregnant rabbit control horns. These results may be due in whole or in part to the leukocytes present in the lumens of the sensitized horns at the time the live organisms were introduced, at which time the previously challenged horns contained considerable amounts of pus.

Summary

A difference was found in the number of bacteria recovered from previously inoculated uteri of estrous and pseudopregnant rabbits which could not be accounted for by differences in the properties of the uterine secretions as culture mediums. The numbers of live *Escherichia coli* recovered at the end of four hours from intact uterine horns was a mean log of 6.54 for the estrous and 8.3 for the pseudopregnant. The difference between these was greater ($P<0.01$) than the difference found in completely excised horns of 8.22 for the estrous and 8.37 for the pseudopregnant.

When heat-killed *Esch. coli* were injected into the lumen of one uterine horn of estrous and pseudopregnant rabbits to stimulate the defense mechanism and, 15 hours later, live *Esch. coli* were introduced into both horns of each rabbit, the mean log numbers of live *Esch. coli* recovered from the four classes of uterine horns after four hours were: sensitized estrous, 4.48; control estrous, 6.76; sensitized pseudopregnant, 6.01; and control pseudopregnant, 8.32.

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Furazolidone for Infectious Enterohepatitis (Blackhead) of Turkeys

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FURAZOLIDONE,[7] formerly furoxone,[3,4,6,7,29] is of increasing interest as new uses for it are found. This paper reports experiments with furazolidone for the prevention and treatment of infectious enterohepatitis of turkeys, together with preliminary type studies of the effect of the drug on normal turkeys.

LITERATURE

The role of furazolidone, N-(5-nitro-2 furfurylidene)-3-amino-2-oxazolidone, in the treatment of various Salmonella infections has been investigated.[6,9-10,13,15,21-23,28] Its antimicrobial properties have been studied.[26] Although sensitive to furazolidone *in vitro*, experimental *Erysipelothrix rhusiopathiae* infection in chicks was not controlled when the drug was administered orally.[27] The drug has little or no value for control of fowl cholera.[1,20] Coccidiostatic activity has been reported for chickens,[11] but it is not an effective coccidiostat for turkeys.[4] Limited data on its activity as a histomonastatic agent have been published[12,18] and it appears to be effective in the control of hexamitiasis in turkeys.[16] It has been reported that there is no adverse effect from continuous feeding to turkey breeders, using egg production, fertility, hatchability, and weight of progeny as criteria.[19] Levels of 100 Gm. per ton of feed had no apparent effect on production.[1] Furazolidone has also been studied to determine its growth-promoting possibilities.[7,10,14,17] The use of the drug in turkeys for the treatment of Salmonella infections and as a histomonastat has received attention in the lay press.[2,5,25]

MATERIALS AND METHODS

Broad Breasted Bronze turkeys of both sexes were used in all experiments unless otherwise stated. With the exception of experiment 7, which required large pens, the turkeys in prevention and treatment studies were confined in pens, approximately 35 ft. square, in which deaths due to infectious enterohepatitis had occurred during the present or previous season. Housing of turkeys in remaining trials will be indicated under individual experiments. The basal ration fed to poults was a commercial turkey developer mash. In addition, whole grains comprised of a mixture of oats and corn or milo were routinely offered free choice to poults when they reached 8 weeks of age. Breeders received a basal ration of 50 per cent commercial breeder mash and 50 per cent whole grains. Furazolidone was added to the ration as an 11 per cent commercial premix, or in a premix prepared from the basal ration in the case of experiments 8 to 12. Restrictions of routine grain feeding are noted in individual experiments. Mortality records were used to evaluate the efficiency of the drug. Criteria used to judge the effect of the drug on normal turkeys were group feed consumption and individual body weights at the start and end of treatment. In the case of breeders, egg production, fertility, and hatchability records were also used.

For convenience of reporting and reading, experiments numbered consecutively are grouped according to their primary nature of prevention, treatment, or effect on normal turkeys.

PREVENTION STUDIES

Experiment 1.—A group of 169 poults were removed from the brooder house when 6 weeks old and distributed at random into four pens. The poults in two of the pens were fed 0.02 per cent of furazolidone in the basal ration for 19 days. The poults in the other two pens received no furazolidone but were otherwise handled identically.

Experiment 2.—This was a repetition of experiment 1 except that 197 poults were started at 7 weeks of age and treatment was for 21 days.

Experiment 3.—This was also a repetition of experiment 1 except that 145 poults were started at 8 weeks of age and treatment was for 22 days.

Results, Experiments 1 to 3.—No death due to infectious enterohepatitis occurred during treatment in any pen receiving 0.02 per cent furazolidone in the basal ration, while 3 controls died in experiment 1, 1 in experiment 2, and none in experiment 3. There were obviously sick and moribund birds in several control pens, but not in treated pens. Therefore, mortality data (table 1) were collected for two 14-day periods after the end of treatment. The average interval from time of exposure to the first death in treated pens was 36.3 days, in control pens, 24.8 days. This is

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TABLE 1—Accumulative Mortality from Infectious Enterohepatitis in 6-, 7-, and 8-Week-Old Poults Given 0.02 per Cent Furazolidone in the Basal Ration* (Mash) at Time of Exposure to Contaminated Ground

Experiment No.	Age of poults (wk.)	No. of days		No. died/No. in pen (%) during post-treatment period	
		To first death (av.)	Treated	14 days	28 days
1	6	19	None	7/43	12/43
1	6	18	None	21/41	29/41
2	7	14	None	7/47	8/47
2	7	25	None	4/51	11/51
3	8	41	None	0/36	4/36
3	8	25	None	3/36	3/36
		(24.8)	.....	42/254(16.5)	67/254(26.4)
1	6	46	19	0/42	1/42
1	6	30	19	1/43	2/43
2	7	35	21	2/47	13/47
2	7	39	21	0/52	6/52
3	8	34	22	2/38	5/38
3	8	34	22	2/35	5/35
		(36.3)	.....	7/257(2.7)	32/257(12.4)

* All poults were offered whole grains when 8 weeks old; therefore, total intake of furazolidone was reduced to the extent that grain was consumed.

indicative of effective prevention during treatment. At 14 days post-treatment, the number of deaths reached 16.5 per cent of the untreated controls and 2.7 per cent of the birds that had received furazolidone. At 28 days post-treatment, the corresponding figures were 26.4 per cent and 12.4 per cent, respectively.

Experiment 4.—Poults in three successive weekly hatches were separated at random into two equal groups, when 8 weeks old, and placed in two heavily infected control pens used in experiment 1. To the basal ration of the poults in one pen, 0.02 per cent furazolidone was added. The others received no furazolidone. After 43, 35, and 28 days of treatment of the respective hatches, the birds and feed were switched from one pen to the other. The same birds continued to receive furazolidone for an additional 56 days. This switch was to provide additional challenge to the furazolidone-treated poults by exposure to heavily contaminated ground.

Results, Experiment 4.—The birds and feed were switched from one to the other pen when the oldest group had received furazolidone treatment for 43 days and the younger groups for 35 and 28 days, respectively. No deaths (table 2) had occurred in the treated pen at this time and the birds appeared in excellent health. In contrast, 15.8 per cent of the controls had died, and many were sick. The characteristic sulfur-colored droppings of infectious enterohepatitis which littered the ground of the control pen were conspicuously absent in the treated pen. On the seventy-third day of the experiment (30 days after switching pens), one death and a loss of the robust appearance of the birds in the

TABLE 2—Accumulative Mortality from Infectious Enterohepatitis in Poults from Three Consecutive Weekly Hatches Exposed in the Same Contaminated Pens when 8 Weeks of Age and Simultaneously Treated with Furazolidone (Experiment 4)

Treatment	Days to first death* (pen av.)	No. died/No. started at days after exposure (pen %)		
		1st period to 43rd day†‡	2nd period to 73rd day†	3rd period to 99th day†
0.02% furazolidone in basal ration; grain free choice	76	0/20 at 43	0/20 at 73	2/20 at 99
	62	0/22 at 35	1/22 at 65	1/22 at 91
	77	0/15 at 28	0/15 at 58	1/15 at 84
Total	(71.7)	0/57(0)	1/57(1.8)	4/57(7.0)
None	21	6/20 at 43	10/20 at 73	10/20 at 99
	32	1/22 at 35	5/22 at 65	7/22 at 91
	26	2/15 at 28	3/15 at 58	5/15 at 84
Total	(26.3)	9/57(15.8)	18/57(31.6)	22/57(38.6)

* From time of exposure of respective age group.

† As of forty-third, seventy-third, and ninety-ninth day of experiment; because of weekly additions to pens, younger groups were exposed for eight or 14 days less than the oldest group.

‡ Turkeys and feed in treated pen were changed to control pen on this day as an additional challenge. Control turkeys and feed were placed in vacated treated pen.

treated pen indicated that the desired additional challenge by exposure to the heavily contaminated control pen had been achieved. However, the turkeys were not seriously ill. Meanwhile nine more deaths for a total of 31.6 per cent had occurred in the control pen. By the end of the experiment (99 days), 7.0 per cent of the treated birds and 38.6 per cent of the untreated contols had died. The daily consumption of the basal ration containing 0.02 per cent furazolidone was reduced considerably in total feed intake by the end of the experiment because of the normal shift to greater grain consumption that occurs in older poults.

TREATMENT STUDIES

Experiment 5.—One hundred and thirty-nine turkeys, 13 and 14 weeks old, in which infectious enterohepatitis was well established were randomly placed into four pens. The turkeys in one pen received 0.02 per cent furazolidone for 18 days, the others for 19 days. Turkeys in the two control pens were handled similarly, except that they received no furazolidone. Grain was withheld for the first eight days of treatment.

Results, Experiment 5.—Treatment of the turkeys in two pens with 0.02 per cent of furazolidone in the basal ration for 18 or 19 days (table 3), with grain withheld for the first eight days, resulted in the death of 2 of 42 birds in one pen; none of 32 birds in the other. Deaths due to infectious enterohepatitis in the two treated pens occurred on the sixth and nineteenth day of treatment, a mortality of 2.7 per cent; in two control pens, the mortality totaled 16.9 per cent. Deaths continued in the control pens after the end of the experiment.

TABLE 3—Mortality Due to Infectious Enterohepatitis in 13- and 14-Week-Old Infected Turkeys Treated with 0.02 per Cent Furazolidone in the Basal Ration* (Experiment 5)

Pen No.	No. of days: To last death	No. of days: Treated	No. died/No. in pen (%)
11	19†	None	6/32
19	19	None	5/33
Total			11/65(16.9)
32	19‡	19	2/42
33	No deaths	18	0/32
Total			2/74(2.7)

* Grain was withheld from both pens for the first eight days of treatment.

† Deaths continued after termination of experiment.

‡ Lesions of infectious enterohepatitis present; enlarged hemorrhagic spleen and other lesions indicated an undetermined intercurrent condition. Other death in this pen occurred six days after treatment started.

Experiment 6.—A group of 166 turkeys, 3 months old, from an infected pen were divided at random into six pens. Two pens of turkeys were untreated controls, two were given 0.011 per cent furazolidone, and two were given 0.0165 per cent furazolidone in the basal ration. No grain was fed to birds in any pen during the two-week period that the drug was fed to those in treated pens.

TABLE 4—Mortality from Infectious Enterohepatitis in 3-Month-Old Infected Poults During Furazolidone Treatment (Experiment 6)

Pen No.	No. of days: To last death	No. of days: Treated	Furazolidone in basal ration (%)*	No. died/No. started (%)
40	14	None	None	10/28
42	14	None	None	9/28
Total				19/56(33.9)
43	14	14	0.011	4/27
45	10	14	0.011	5/28
Total				9/55(16.4)
41	6	14	0.0165	1/28
44	8	14	0.0165	5/27
Total				6/55(10.9)

* No grain fed to any pen during the treatment period.

Results, Experiment 6.—Furazolidone, at the 0.0165 per cent level, stopped losses within eight days (table 4) after a loss of 10.9 per cent. At the 0.011 per cent level, it was less effective, but it did stop losses in one pen by the tenth day and reduced the average losses in the two pens to about one half (16.4%) of that of the controls in which death losses had not been stopped and which suffered an average loss of 33.9 per cent for the two-week period.

Experiment 7.—A group of 252 infected poults, 3 to 4 months old, were randomly distributed into two large pens and were fed furazolidone at the 0.011 per cent level in the basal ration, which was pelleted. No grain was fed during the 12 days of treatment. The poults in one pen were taken off treatment and those in the other placed on the 0.0165 per cent level of furazolidone with grain offered free choice.

Results, Experiment 7.—When 0.011 per cent furazolidone was fed in the basal ration as the only feed (table 5), losses were checked in nine days, with an average loss of 5.1 per cent. Since some birds were extremely sick or moribund when treatment started, losses were anticipated. As a result of feeding 0.0165 per cent furazolidone in a pelleted basal ration to birds in

TABLE 5—Mortality from Infectious Enterohepatitis in a Mixed Group of 3- to 4-Month-Old Infected Turkeys During Furazolidone Treatment (Experiment 7)

Pen No.	No. of days to last death*	Treatment	No. died/ No. in pen (%)
51	9	0.011% furazolidone in basal ration† for 12 days	9/126
52	7		4/126
Total			13/252(5.1)
51	43	0.0165% furazolidone in basal ration‡ from 13th to 76th day	1/116(0.9) (1 accidental death occurred)
52	69	None 13th to 76th day‡	11/122(9.0)

* From start of experiment.
† No grain fed.
‡ Grain fed.

one pen, with grain fed free choice for the balance of the season, only 1 bird (0.9%) died of infectious enterohepatitis up to market time. In the control pen, 11 of 122 birds (9.0%) died in the corresponding period.

Effect on Normal Turkeys

Experiment 8.—A group of 40 poults was divided at random into two groups and placed in small brooder houses at 7 weeks of age. Those in one house received 0.02 per cent furazolidone in the basal ration for three weeks; the others received an identical ration without furazolidone.

Experiment 9.—Forty poults, 13 weeks old, were distributed at random into eight wire-bottomed cages. Those in every other cage received 0.02 per cent furazolidone in the basal ration for three weeks. No grain was fed.

Experiment 10.—This was a repeat of experiment 9, using 48 poults, 8 weeks old, with 6 birds in each cage.

Experiment 11.—This was also a repeat of experiment 9, using 40 turkey hens, 23 and 24 weeks of age.

Results, Experiments 8 to 11.—A few losses due to picking occurred. However, the data (table 6) indicate that the 0.02 per cent level of furazolidone fed to poults, 7 to 24 weeks old, with or without grain did not adversely affect their weight gains or feed consumption. Hence, these criteria gave no evidence of toxicity.

Experiment 12.—Turkeys nearing production age were randomly distributed into four pens, so that each had 10 females and 2 males. Equal parts of the basal ration and whole grains were mixed and furazolidone (0.02%) was added and fed in two of the pens for 35 days. The birds in the other two pens received the basal ration-grain mixture only. They came into production while on treatment.

Results, Experiment 12.—Detailed records kept on 10 breeder turkey hens and 2 toms in each of two pens showed no important differences in fertility, hatchability, feed consumption, or body weights as a result of feeding furazolidone (0.02 %) when compared with two like pens of controls. Since similar findings were recently reported,[19] these records are omitted here.

TABLE 7—Effect of 0.02 per Cent Furazolidone on Sexual Maturity of Turkey Breeders as Measured by Date of First Egg (Experiment 12)

No. of turkeys (females)	Treatment	Days after start of experiment first egg was laid
10	Furazolidone	24
10	Furazolidone	29
Av.		26.5
10	None	10
10	None	12
Av.		11

An interesting finding, however, is (table 7) that sexual maturity, measured by start of egg production, was delayed in each treated pen.

Discussion

The prophylactic experiments herein reported were dependent on infection by exposure to contaminated ground. While this simulates the method of acquiring natu-

TABLE 6—Effect of 0.02 per Cent Furazolidone on Normal Turkeys of Various Ages

Experiment No.	No. of turkeys started in each treatment (age in wk.)	Av. gain or loss (−) in lb. by treatment and sex					
		0.02% furazolidone			Controls		
		Males	Females	Mixed	Males	Females	Mixed
8*	20 (7 wk.)	....	....	2.6	....	....	2.5
9	20 (13 wk.)	1.7	1.3	....	1.2	1.1	....
10	24 (8 wk.)	2.7	2.1	....	2.7	1.8	....
11	20 (23 and 24 wk.)	....	−0.1	....	....	−0.1	....
Av.		2.2	1.65	2.6	1.95	1.4	2.5

* Birds were on litter and received grain when 8 weeks old. All others were on wire-bottomed cages; no grain fed. Feed consumption, remarkably uniform in all experiments, was slightly higher in groups receiving furazolidone, except in experiment 11. Furazolidone was fed for three weeks.

ral infection, it also introduces some uncertainty that treated and control pens were equally exposed. This may account for the variation in the day of the first death among some duplicate control pens (table 1). However, in experiments 1 to 4, which were prevention studies, reduced mortality and the increased interval from exposure to first death in treated birds as compared with those in control pens indicate that 0.02 per cent furazolidone in the basal ration, with grain offered free choice at 8 weeks of age, did prevent excessive losses from infectious enterohepatitis. Visual inspection of all pens during the experiment confirmed this conclusion, since the treated birds appeared normal, or nearly so, while the haggard appearance of the controls attested to their infection.

The 0.02 per cent level of furazolidone, with grain withheld for the first week, checked an outbreak in turkeys, 13 to 14 weeks old, in experiment 5. This level, therefore, will treat as well as prevent infectious enterohepatitis.

At the 0.0165 per cent level in the basal ration, with no grain, furazolidone was effective in checking a severe outbreak in eight days in experiment 6. The 0.011 per cent level was less effective, but losses were reduced to about one half of those in the controls which received no treatment. The majority of deaths among birds in this experiment which were treated with the 0.0165 per cent level occurred early in the treatment period. Since the birds were thin when examined, most of the deaths represented cases with well-advanced infection before treatment started. In experiment 7, on the other hand, 0.011 per cent furazolidone in the basal ration with no grain being fed stopped an outbreak which, as indicated by mortality data in table 5, was relatively mild. Thus, it is indicated that a level of 0.011 per cent is too low for treatment, except in mild infections, but the results suggest it can be used as a preventive level. If losses increase at this level, the 0.0165 per cent or the 0.02 per cent level in the total ration would be indicated until losses are checked.

As noted in experiment 4, turkeys fed the basal mash and offered grain free choice normally shift from an initial low to a high ratio of grain intake in relation to mash. This operates to reduce the total amount of drug in the total feed intake and must be constantly borne in mind when evaluating or computing levels.

As has been reported for lower levels,[1,19] there was no indication of any detrimental effect of furazolidone on 7-week-old poults at the 0.02 per cent level, with grain added free choice at 8 weeks of age, nor in poults 8, 13, or 23 and 24 weeks old without grain. When fed at the same level in the total ration starting prior to production and continuing for 35 days, no detrimental effect on breeders other than a delay in coming into production was observed. This finding has not been observed in the field where levels lower than 0.02 per cent are generally fed for shorter intervals.

CONCLUSIONS

1) When furazolidone, at the 0.02 per cent level in the mash, was fed with free access to grain at 8 weeks of age, excessive losses did not occur from infectious enterohepatitis in poults exposed to contaminated ground at 6 to 8 weeks of age. This was shown by the low mortality and the greatly increased interval from time of exposure to time of death in treated birds.

2) With furazolidone at levels of 0.0165 and 0.02 per cent of the total feed, deaths due to infectious enterohepatitis in birds in infected pens usually were checked within eight days.

3) Furazolidone, at the 0.011 per cent level in the basal ration as the only feed, checked mild infectious enterohepatitis but, in apparently severe exposures, higher levels were more effective.

4) Furazolidone, at the 0.02 per cent level, produced no discernible detrimental effect on normal turkeys over 7 weeks old, except that sexual maturity was delayed when it was fed to breeders approaching the start of egg production.

ADDENDUM

After this report was prepared for publication, the paper of Horton-Smith and Long* appeared. These workers tested furazolidone at concentrations of 0.015 to 0.04 per cent against histomoniasis (infectious enterohepatitis) induced by administration of infective Heterakis ova. They record some interesting observations and deductions. In comparable experiments,

* Horton-Smith, C., and Long, P. L.: Furazolidone in the Control of Histomoniasis (Blackhead) in Turkeys. J. Comp. Path. and Therap., *66*, (1956): 22–34.

their findings are similar to those reported in this paper.

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The Toxicity of Malathion in Chickens

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VARIOUS new insecticides have been introduced recently in veterinary medicine. Among these are the organic phosphorus compounds of which malathion (0,0-dimethyl dithiophosphate of diethyl mercapto-succinate) is a member. This chemical has been used as a spray for control of insect pests on plants and for fly control in stables and barns. Bell *et al.*[1] reported on the toxicity of this drug in dogs. The only discomfort produced in dogs by giving malathion, 3,500 mg. per kilogram of body weight orally, was vomiting within 35 minutes after administration. The dogs which were anesthetized immediately after administration retained the drug and showed no signs of toxicity after recovery from the anesthesia. Dipping of dogs four times in a 2 per cent solution of malathion at four-day intervals was also harmless. As a result of these toxicity experiments, malathion in the above concentration was recommended as a safe insecticide to use on dogs and in flea breeding places frequented by dogs.[2]

Rădeleff *et al.*[3] reported that the minimum oral toxic dose for baby calves appears to be between 10 and 20 mg./kg. They reported, however, that sheep tolerated 50 mg./kg. doses but were poisoned by 100 mg./kg. and all higher doses. Golz and Shaffer[4] reported that in chickens there were no deaths and they appeared normal after consumption of feed containing 100 and 1,000 p.p.m. for ten weeks. The average daily intake was calculated to be 7 mg./kg. and 90 mg./kg., respectively, of the 95 per cent technical malathion. At the 5,000-p.p.m. level, or an average consumption of 450 mg./kg./day, these investigators found that chickens showed definite signs of toxicity, such as retardation of growth, slow feather development, soft droppings, weakness of legs, and paralysis.

Because of the possibility of toxic effects occurring in livestock and chickens on farms where malathion is used, the following experiments are reported.

MATERIALS AND METHODS

Experiment 1.—On Jan. 14, 1956, 166 genetic top cross, White Leghorn chickens, 1 day old, obtained from the Poultry Department at A. & M. College of Texas, were placed in poultry batteries. They were fed a balanced, commercial starter ration. When 3 weeks old, they were separated into three groups according to their weights and banded for identification. Each of the 55 birds in group 1 was given a single dose of 100 mg./kg. of malathion by means of a tube passed into the crop. The 55 chickens in group 2 were dusted once a week for four weeks with 4 per cent malathion powder. The 56 birds in group 3 were kept as controls. The malathion solution given to group 1 was made from a commercial stock solution containing 25 mg. per cubic centimeter. The dusting powder was a commercial malathion preparation containing 4 per cent pure malathion in a wettable powder.

Experiment 2.—The chickens used in groups 1 and 3, in experiment 1, were used again a week later in the second experiment. Groups 4 and 5 were subdivisions of group 1 and contained 27 chickens each. Groups 6 and 7 were subdivisions of group 3 and contained 28 chickens each. On Feb. 11, 1956, when they were 4 weeks old, group 4 chickens were given 100 mg.; group 5, 200 mg.; group 6, 400 mg.; and group 7, 600 mg./kg. of malathion each orally.

Experiment 3.—The 21 fowls used in this experiment were White Leghorn hens, 1 year old, secured from the Poultry Farm at A. & M. College of Texas. Their average weight was 4.61 lb. Each of the 10 birds in group 8 was given 100 mg./kg., and each of the 11 in group 9 were given 200 mg./kg. of malathion orally.

RESULTS

None of the chickens in experiment 1 (table 1) showed any signs of toxicity. The death of 1 chicken in group 1 was attributed to other causes. The food consumption of the orally treated chickens fell for the first 12 hours but soon returned to normal. The chickens which were dusted showed no signs of toxicity during the four-week period.

In experiment 2 (table 2), the chickens in group 4, which were given 100 mg./kg.

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TABLE 1—Effect of Malathion on 3-Week-Old Chickens

Group No.	No. of chickens	Av. wt. at 3 weeks (Gm.)	Medication	No. of deaths
1	55	222.66	100 mg./kg. malathion orally	1
2	55	222.68	4% malathion dusting	None
3	56	221.75	Controls	None

of malathion twice within a period of seven days, showed no signs of toxicity. One died within 16 hours from the time the drug was administered but its death was attributed to factors other than malathion poisoning.

The chickens in group 5 were given 200 mg./kg. seven days after the first dose of 100 mg./kg. in experiment 1. Of the 27 chickens, 4 died within three hours after the second dose of malathion was administered, another died within six hours, and the rest recovered after showing varied degrees of toxicity.

The chickens of group 6 (400 mg./kg.) showed signs of toxicity within two hours after the administration of the malathion (their first dose). Of the 28, 8 died within three hours, 13 died between three and six hours, and 5 died between six and 16 hours after the administration of the malathion. The other 2 birds showed severe signs of poisoning for two days, then recovered.

Of 28 birds in group 7, given 600 mg./kg. of malathion as their first dose, all developed severe signs of toxicity within two hours and all died within 16 hours; 13 died within three hours, 14 between three and six hours, and 1 between six and 16 hours from the time of the administration of the drug.

In experiment 3 (table 3), in which the chickens were 1 year old, the results varied in many respects from those obtained with the younger birds. All the chickens in groups 8 and 9 showed varied degrees of toxicity from the malathion within three to 16 hours. In group 8 (100 mg./kg.), 1 of the 10 chickens died within 16 hours of medication. In group 9 (200 mg./kg.), 1 died within three hours and 5 died in six to 16 hours. The other 5 birds survived, but the period of convalescence was long (1 was still lame after 3 weeks).

TABLE 2—Effect of Malathion on 4-Week-Old Chickens

Group No.	No. of chickens	Av. wt. at 3 weeks (Gm.)	Medication	No. of deaths			
				3 hr.	3 to 6 hr.	6 to 16 hr.	Total
4	27	301.36	100 mg./kg. orally	None	None	1	1
5	27	305.33	200 mg./kg. orally	4	1	....	5
6	28	307.31	400 mg./kg. orally	8	13	5	26
7	28	313.83	600 mg./kg. orally	13	14	1	28

The signs of toxicity with malathion shown by the chickens in the poisoned groups included drowsiness, incoordination, reluctance to move, assuming a sitting position on the hocks, excessive salivation with mucus hanging from the beaks, slight blueness of the skin, diarrhea, brownish droppings tinged with blood, coma, and death. At no time did the chickens show signs of nervous excitability or uneasiness. On necropsy of the chickens which died during these experiments, no lesion was consistently found that could be considered as caused by the malathion. The lesions frequently encountered were severe congestion of the heart and a darker color of the heart muscle, especially the apex. Many birds showed dilation of the subcutaneous blood vessels, resulting in the carcass having a bluish color.

DISCUSSION

These experiments indicate that malathion toxicity varies according to the route of administration. No signs of toxicity re-

TABLE 3—Effect of Malathion on 1-Year-Old Chickens

Group No.	No. of chickens	Av. wt. at 1 yr. (Gm.)	Medication	No. of deaths			
				3 hr.	3 to 6 hr.	6 to 16 hr.	Total
8	10	2095.63	100 mg./kg. orally	None	None	1	1
9	11		200 mg./kg. orally	1	None	5	6

sulted when chickens were dusted weekly for four weeks with a 4 per cent malathion powder; their food consumption was not altered. This suggests that malathion might be used effectively against lice, "stick-tight" fleas, and mites of chickens, but more controlled investigations are needed before conclusions can be drawn.

The $l.d._{50}$ of malathion in the feed, as reported by Golz and Shaffer [4] (above 850 mg./kg.) appears to be too high. From the experiments reported here, it is evident that young chickens can withstand more malathion by oral administration than can older birds. The $l.d._{50}$ for young chickens 3 weeks of age seems to be between 200 and 400 mg./kg. and, for the older fowls (1 year old), between 150 and 200 mg./kg. It is also of interest that almost all the poisoned birds recovered if they survived the first 16 hours. This may indicate that: *(a)* the drug could have had a high rate of excretion or *(b)* it could have been rapidly detoxified or, possibly, counteracted by some unknown factor or factors in the body.

Since the malathion action in poisoned birds has been attributed to cholinesterase inhibition, it is of interest that the chickens in these experiments developed signs of parasympathomimetic activity, except for the nervous irritability that is usually associated with this action. Whether this manifestation is completely absent in chickens, or is of such a transient nature that it is not noticed, is a matter of speculation. During the time that toxic signs occurred in the older chickens, it was noticed that many of them voided *Ascaridia galli*, the roundworm of chickens, with their droppings. This, however, was attributed only to the parasympathomimetic action of the malathion on the intestine. Many worms were found in the intestine of dead birds upon necropsy.

SUMMARY

Experiments on malathion toxicity to chickens by oral dosage and dusting were performed on 297 birds. Birds given 4 per cent dust treatment four times at weekly intervals did not develop signs of toxicity.

In these experiments, younger birds (3 weeks old) apparently were more tolerant to the effects of malathion at the 200 mg. per kilogram of body weight level than were older birds (1 year old).

The signs of poisoning with malathion in chickens are described.

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Comparative Histopathology of Skin Reactions in the Chicken, Turkey, and Canary Infected with a Strain Variant Canary Pox Virus

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THE PREDOMINANT FEATURE observed in the diseases produced by the viruses of the pox group is the papular cutaneous eruption. A filterable agent reportedly isolated from a human skin papilloma,[1] and cultivable on the chorioallantoic membrane of the embryonating hen's egg, was identified as a strain of avian pox virus when inoculation of the agent into defeathered chicken skin gave rise to pocklike lesions.[2] Further host range studies revealed that the virus produced lesions in the turkey and canary in addition to the chicken, but failed to do so in quail, pheasants, pigeons, and ducks.[2,3] A histological study was undertaken to determine the response of cutaneous epithelium to infection in each of the three susceptible species. The results of this study are reported herein.

PROCEDURE

The virus employed was contained in a chorioallantoic membrane suspension originally received from Dr. J. A. Bivins and serially egg-passaged some 18 times in this laboratory. Stock virus was prepared from the chorioallantoic membranes of fertile White Leghorn eggs infected 96 hours previously. About 10^8 infective viral particles, in 0.1-ml. amounts, were inoculated on to the chorioallantois of 10-day-old eggs,[4] and the eggs were then incubated at 36 C. The membranes which showed confluent lesions at 96 hours were harvested by removing only that part of the membrane at the base of the artificial air sac. This material was ground in a mortar and pestle with sterile sand and 1.0 ml. of medium 199[5] per membrane, and then centrifuged at 2,000 r.p.m. for ten minutes. The resulting supernatant, which represented the stock suspension of virus, was considered to be undiluted. It was routinely stored in sealed glass ampules at —70 C.

The chickens employed in this study were from the Single-Comb White Leghorn flock[6] maintained by the Division of Poultry Husbandry of the University of California at Berkeley. The flock originated in 1933 and, during the whole period of observation, there has been no history of diseases of the pox group. Male turkeys, 7 weeks old, and female Gluck Roller canaries, 6 months old, were used. They were obtained from a commercial source. Defeathered skin areas were scarified with a scalpel, and undiluted virus suspension then was applied by means of a stiff-bristled brush.

For histological examination, skin lesions were removed by biopsy on the sixth day following viral inoculation. The tissues were fixed in 10 per cent formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

RESULTS

In chickens, discrete papules appeared in inoculated areas usually in four to six days. Lesions rarely became confluent, were never observed to become pustular or to be accompanied by scab formation, and were generally completely healed ten to 14 days after their appearance. The course of the infection in turkeys was almost identical. In the canary, the lesions

Fig. 1—Normal chicken skin. x 200.

From the Virus Laboratory, University of California, Berkeley.

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became confluent, resulting in scab formation followed by death in 19 to 20 days after inoculation.

Sections are shown through normal chicken skin (fig. 1) and through the lesions in the inoculated chicken (fig. 2). The lesions in chicken and turkey skin were characterized by a marked inflammatory reaction in the corium and deeper layers of the subcutaneous connective tissue, while the epithelium itself showed only slight thickening. The inflammatory reaction in the subcutaneous tissue was severe, however, with marked congestion and edema. The most outstanding lesion was a heavy infiltration of inflammatory cells extending to a depth of 2 to 3 mm. beneath the epithelium. Most of these cells were of the mononuclear type with a few polymorphonuclear cells being seen. There was evidence of nuclear degeneration characterized by pyknosis in many of the inflammatory cells. No inclusion bodies were observed.

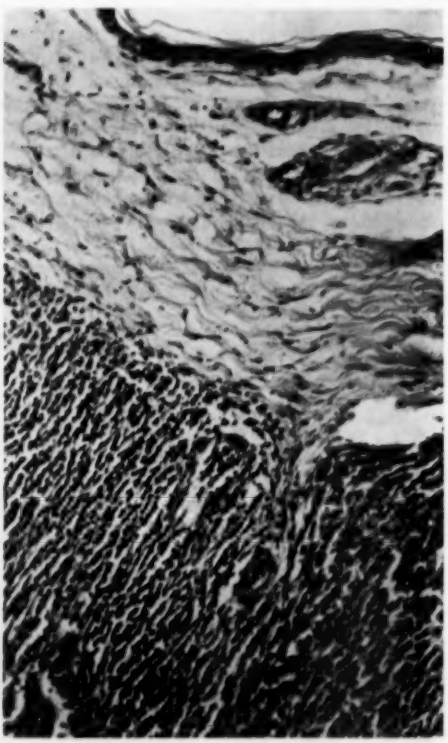

Fig. 2—Infected chicken skin showing subcutaneous infiltration but normal epithelium. x 200.

The skin sections from infected canaries (fig. 3) showed marked changes in the epithelium as well as in the subcutaneous tissues. The epithelium of the affected areas manifested a marked thickening, which was most pronounced at the centers of these areas, tapering off gradually to normal thickness at the peripheral regions of the lesions. There was some increase in the stratum corneum, but most of the cell proliferation was in the stratum malpighii. The cells within the area of thickened epithelium showed evidence of degeneration which varied from hydropic change to complete nuclear disintegration. Many of these degenerating cells contained eosinophilic inclusion bodies (fig. 4). The smaller bodies appeared to be homogeneous in structure, while the larger inclusions appeared vacuolar with the periphery staining more intensely than the center. Some of the largest inclusions exhibited a granular appearance, suggesting that they might be composed of clumps of elementary bodies. The spectrum of inclusion bodies resembled closely that described by Burnet and Barnard [7] in the skin lesions induced by the Kikuth canary pox virus.[8]

In the dermal layers, immediately below the epithelium, a severe inflammatory reaction with marked congestion and edema was observed. There was profuse cellular infiltration composed chiefly of mononuclear cells with a few polymorphonuclear cells. There was also considerable clumping of chromatin material suggesting nuclear degeneration. The numerous cytoplasmic inclusions described by Burnet and Barnard,[7] however, were not observed. This severe infiltration carried to a depth of about 2 to 3 mm. beneath the basal layer of the epidermis. In the deeper layers of subcutaneous connective tissue below the severely infiltrated zone, there was marked diffuse edema and some congestion. The infiltration with inflammatory cells, although diffuse, was much less dense than in the more superficial layers of the subcutaneous tissues. The cytoplasm of many of the macrophages was vacuolar, but no inclusion bodies were seen. The inflammatory reaction of the subcutaneous tissues was generally more extensive than that of the epithelium and was observed

to extend laterally beneath regions of normal epithelium.

Discussion

On the basis of lesions produced in the skin of chickens, turkeys, and canaries, this agent was previously classified as a tripathogenic strain of avian pox.[2,3] The present observations have elucidated, further, that the lesions produced are less severe in chickens and turkeys than in canaries. In the chicken and turkey, the predominant pathological feature was a marked inflammatory reaction in the corium and deeper layers of the subcutaneous connective tissue, while the epithelium itself remained essentially normal. In contrast, infected canary skin manifested marked changes in the epithelium as well as in the subcutaneous tissues. There was massive epithelial hyperplasia with the formation in the cytoplasm of numerous eosinophilic inclusion bodies.

The skin lesions induced in the chicken and turkey were relatively mild and transient, seldom progressing beyond the papular stage and completely healing within three weeks following viral inoculation. In the canary, the lesions became confluent and showed marked scabbing, followed by death at the end of the third week. Thus, greater virulence of the virus for the canary, as manifested by the intracytoplasmic inclusions induced in the parasitized susceptible cells, was substantiated by the observed gross reactions.

Such differences, as observed here, in the ability of this strain of virus to infect different species, and the extent of this infection, are characteristic of the bird-pox diseases, as well as of the mammalian poxes, and are difficult to explain. Since infection with this virus was demonstrated previously to be influenced by the age of the host,[2] all birds used were 6 months old or younger. Other hypotheses which might be invoked to explain this phenomenon are the genetic factors and those of hormonal balance in the various species. It should be noted that the virus was incapable of producing any lesions in quail, pheasants, pigeons, and ducks. Some investigators have suggested that cytoplasmic inclusions were most readily formed in cells which suffered least from the injurious effects of virus multiplication.[9]

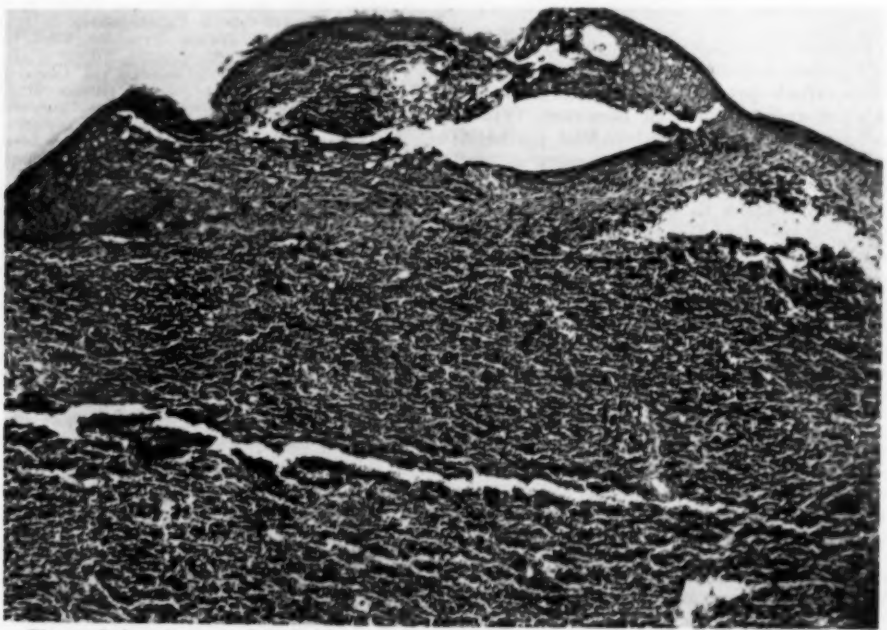

Fig. 3—Infected canary skin showing vesicle surrounded by hydropic epithelial cells. There is also a marked subcutaneous infiltration. x 120.

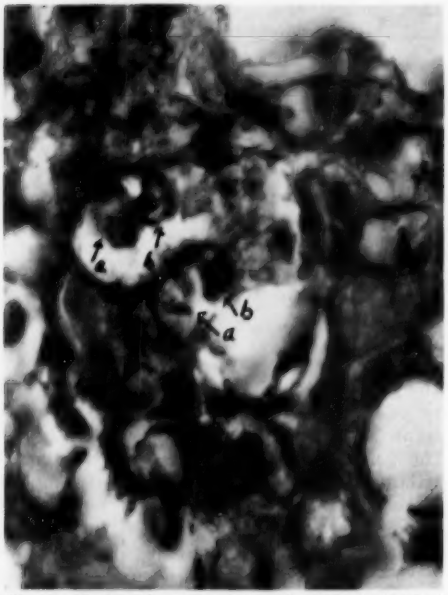

Fig. 4—Infected canary skin showing individual cells containing intracytoplasmic inclusions (a). These bodies are bright red in stained preparations. The nuclei (b) are pushed to one side of the cell and show pyknosis. x 1,000.

Brunett [10] reviewed the findings with two other tripathogenic canary pox viruses which produced skin lesions in the chicken and pigeon. In one case, typical inclusions were observed in the epithelial cells of the chickens and pigeons as well as of the canaries; while in the second case, the lesions in chickens and pigeons were nonspecific. Durant and McDougle [11] mentioned having observed numerous virus inclusions in the skin sections from canary lesions and turkey lesions but none in the sections from chickens. In the histological studies described here, inclusion bodies were observed only in the cutaneous lesions of the canary.

SUMMARY

A variant strain of canary pox virus, when inoculated into scarified, defeathered areas of the skin of the chicken, turkey, and canary, regularly produced cutaneous papular eruptions. Histological examination revealed the lesions in the chicken and turkey to be characterized by marked inflammation in the corium and subcutaneous connective tissue with the epithelium remaining essentially normal. In the canary, there was massive proliferation of epithelium with numerous intracytoplasmic inclusions, as well as severe subcutaneous lesions. Grossly, the lesions in the chicken and turkey were usually mild and transient, while in the canary the lesions became confluent and formed thick scabs.

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A Comparison of the Effect of Gallogen and Sulfarlem on the Normal Bile Flow of the Cockerel

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GALLOGEN ® * has been shown to retard serum and tissue cholesterol elevations in cholesterol-fed cockerels.[1-6] With man and mammalian experimental animals, several workers have reported [5,7-9] that gallogen is a "true" choleretic, *i.e.*, it causes an increased production of an unattenuated bile, having a normal specific gravity and constitution. In a paper [1] dealing with the ability of gallogen to lower serum cholesterol levels of cholesterol-fed cockerels, it was postulated that its ability to increase the biliary secretion of cholesterol and bile salts might participate in this effect. This communication reports experiments which were designed to evaluate the effect of gallogen on bile secretion by the cockerel and to compare it in this regard with sulfarlem (5-(*p*-methoxyphenyl)-1,2-dithiol-3-thione), which has been reported [2-4] to be an active choleretic.

METHODS

Single-comb, White Leghorn cockerels, 14 weeks old and weighing about 1.2 kg., were used. The birds were placed under mild general anesthesia by the intravenous injection of approximately 30 mg. per kilogram of body weight of napental ® and restrained to a table with the right lateral aspect of the bird facing the operator. The operative site was then prepared for surgery. A 5-cm. incision was made in the abdominal wall beginning just posterior to, and parallel with, the last rib. The duodenal loop lies just under the incision site. The hepatic and cystic bile ducts were exposed by blunt dissection. The hepatic duct was ligated and the cystic duct cannulated with 20-gauge polyethylene tubing, secured with a ligature. The duodenum was returned to the abdominal cavity, which was closed with wound clips (or sutured, in the case of birds used for "chronic" fistulae), leaving the end of the polyethylene cannula exteriorized for hourly bile collection. Sedation was maintained when necessary.

In experiment 1, 5 cockerels were cannulated and bile collection was begun immediately. A three- or four-hour control period was used. Three of the birds were given intravenously 1 ml. of a solution containing 40 mg. of gallogen per milliliter, 1 bird 80 mg. *per os*, and the collection continued.

The volume of the bile samples was measured to the nearest 0.05 ml. and the weight recorded for the total solids remaining after taking to dryness plus three hours at 110 C. Cholesterol was determined by the method of Zlatkis *et al.*[10] in the samples from the intravenously treated birds.

In experiment 2, parallel control birds were substituted for the control period used in experiment 1, to permit a longer observation period after oral administration of the drug.

Five control cockerels were given 10 ml. of water by stomach tube, cannulated, and bile collection began 20 minutes after the dose of water. The volume and total solids of the samples were measured. Five other cockerels were given 80 mg. of gallogen dissolved in 10 ml. of water, and their bile excretion studied exactly as for their parallel controls.

Two of the control birds from experiment 2 were also used for experiment 3, 24 hours after cannulization. At this time, bile secretion was steady in volume and had presumably reached equilibrium with hepatic bile production. Simple restraint, with no anesthesia, was satisfactory here. Two one-hour control samples were collected. Eighty milligrams of gallogen dissolved in 10 ml. of water was given orally at the end of the second control hour. Bile collection was continued for a further five or six hours. The volume and total solids content of the samples were determined as in experiment 1.

In order to compare the effects of sulfarlem with those of gallogen under the same conditions, bile fistulae were produced in 3 cockerels. One bird was used as a control bird, and 2 birds were given orally 3 mg. and 6 mg., respectively, of sulfarlem. Bile was collected for periods up to four hours after the administration of the drug. Volume and total solids content were measured as in the gallogen series.

Two birds were used in which the cystic duct had been cannulated 24 hours previously, as in the gallogen experiment. Two one-hour control samples were collected, after which 15 mg. of sulfarlem was given orally to each of the 2 birds. Bile collection was then continued for a further three or four hours. The samples were measured as before.

From the Research Department, S. E. Massengill Co., Bristol, Tenn.

* The diethanolamine salt of the mono-(+)-camphoric acid ester of α,4-dimethylbenzyl alcohol.

TABLE 1—Effect of Gallogen on Bile Secretion by the Cockerel

Bird No.	Dose	Hours of bile secretion: Control period 1 ml.	1 mg./ml.*	1 chol.†	2 ml.	2 mg./ml.	2 chol.	3 ml.	3 mg./ml.	3 chol.	4 ml.	4 mg./ml.	4 chol.	After gallogen 1 ml.	1 mg./ml.	1 chol.	2 ml.	2 mg./ml.	2 chol.	3 ml.	3 mg./ml.	3 chol.
1	40 mg., intravenously	3.8	50.6	141	1.5	56.7	138	1.2	65.6	98	0.8	64.7	103	2.0	38.8	65	1.2	43.2	125	0.8	54.3	65
2	40 mg., intravenously	0.7	51.9	105	0.4	78.0	113	0.2	137.2	31	....	....	....	1.8	41.0	60	0.8	33.1	35	0.3	71.7	83
3	40 mg., intravenously	1.6	44.3	80	1.4	56.3	118	1.4	52.8	92	....	....	....	2.8	34.5	158	1.2	45.8	87	0.8	59.8	120
4	None	1.1	35.5	....	1.1	23.3	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
5	80 mg., orally	0.8	52.5	....	1.0	42.0	....	0.8	40.0	....	....	....	....	1.1	43.8	....	....	....	....	1.5	40.0	....

* mg./ml. = milligrams of total solids per milliliter of bile. † chol. = milligrams of cholesterol per gram of bile solids.

TABLE 2—Effect of Orally Administered Gallogen on Bile Secretion by the Cockerel

Bird No.	Dose	Hours of bile secretion: 1 ml.	1 mg./ml.*	2 ml.	2 mg./ml.	3 ml.	3 mg./ml.	4 ml.	4 mg./ml.	5 ml.	5 mg./ml.	6 ml.	6 mg./ml.	7 ml.	7 mg./ml.	8 ml.	8 mg./ml.
1	None	2.2	47.1	2.2	47.7	1.2	33.2	1.0	33.7	1.0	29.4	0.7	33.0	0.5	42.4	0.5	36.2
2	None	0.2	65.0	0.3	70.4	0.2	70.5	0.3	70.2	0.7	51.3	0.5	64.1	0.4	66.4	....	....
3	None	1.0	37.3	0.6	46.8	0.4	62.5	0.4	53.3	0.6	38.4	0.4	64.7	....	....	....	....
4	None	2.8	57.8	2.4	47.2	1.4	40.6	1.4	44.8	1.2	34.2	0.8	40.0	0.6	49.8	....	....
5	None	1.0	58.7	2.0	39.7	2.0	32.7	2.6	29.9	1.6	31.8	1.5	30.8	....	....	....	....
6	80 mg.	3.0	26.4	1.8	25.6	1.8	25.8	2.0	24.0	1.6	26.9	1.2	30.5	1.2	24.9	....	....
7	80 mg.	2.7	42.2	1.8	48.0	1.4	32.2	1.4	32.1	1.2	33.1	0.7	41.3	....	....	....	....
8	80 mg.	1.0	46.1	1.5	45.5	1.2	46.5	0.8	48.8	0.7	47.3	0.7	56.4	....	....	....	....
9	80 mg.	3.0	45.7	1.8	56.4	1.1	53.7	0.4	48.3	0.2	57.3	1.0	41.6	....	....	....	....
10	80 mg.	1.9	43.1	1.7	44.8	1.1	44.8	1.0	41.0	1.0	41.7	....	....	....	....	....	....

* mg./ml. = milligrams of total solids per milliliter of bile.

TABLE 3—Effect of Orally Administered Gallogen on Bile Secretion by the Cockerel

Bird No.*	Dose	Hours of bile secretion: Control 1 ml.	Control 1 mg./ml.†	Control 2 ml.	Control 2 mg./ml.	After gallogen 1 ml.	After gallogen 1 mg./ml.	After gallogen 2 ml.	After gallogen 2 mg./ml.	After gallogen 3 ml.	After gallogen 3 mg./ml.	After gallogen 4 ml.	After gallogen 4 mg./ml.
1	80 mg.	1.15	50.2	1.1	27.2	1.3	39.9	1.5	34.1	1.3	37.6	....	....
2	80 mg.	0.9	29.1	0.9	52.7	1.4	25.8	1.35	22.5	2.2	19.6	2.0	20.4

* Birds 1 and 2 were the same birds designated on table 2 as control birds 1 and 2. † mg./ml. = milligrams of total solids per milliliter of bile.

RESULTS AND DISCUSSION

The results of experiments 1, 2, and 3 with gallogen are presented in tables 1, 2, and 3, respectively. Gallogen evidently possesses no significant "true" choleretic activity in the cockerel and, therefore, this action can not be involved in its ability to lower the serum cholesterol of cholesterol-fed cockerels. The small choleretic activity observed is probably a reflection of the obligatory biliary excretion of the drug, comparable to that found with such drugs as sodium salicylate.

Gallogen's hydrocholeretic activity, found only after the intravenous administration of the 40-mg. dose, is slight and of approximately two hours' duration. The biliary cholesterol excretion was unchanged after intravenous gallogen.

The results of the experiments with sulfarlem are shown (table 4). An increase in the biliary volume occurred three to four hours after the oral administration of the drug, a result similar to that reported for dogs.[2-4] An oral dose of 3 mg. is apparently as effective as a 15-mg. oral dose. An unambiguous estimation of the percentage increase is not possible because of individual variation among birds in bile output, and since the volumes involved are so small and also gradually decline in the unmedicated cockerel, it appears to be of the order of 200 to 400 per cent. The decline in bile flow that occurs in unmedicated cockerels is presumably due to the fact that the hepatointestinal recirculation of the bile salts has been interrupted.

TABLE 4—Effect of Sulfarlem on Bile Secretion by the Cockerel

Hour	Bird No. 1* ml.	1* mg./ml.	2* ml.	2* mg./ml.	3† ml.	3† mg./ml.	4† ml.	4† mg./ml.	5† ml.	5† mg./ml.
	(Control)		(Control)		(Control)		(3 mg. orally)		(6 mg. orally)	
1	0.75	52.8	1.0	50.0	2.35	52.7	0.7	65.7	1.0	54.2
2	0.3	60.7	1.25	49.7	1.6	58.7	1.75	62.0	0.8	67.2
	(15 mg. orally)		(15 mg. orally)							
3	0.3	52.4	1.25	38.3	1.30	46.4	1.9	48.6	1.1	57.1
4	0.2	54.1	1.7	34.0	0.75	44.3	0.9	48.3	1.0	46.6
5	0.5	41.6	1.5	35.1	0.8	39.7	1.3	46.4	0.6	40.6
6	0.9	41.1	1.2	41.1	0.7	38.3	1.0	56.3	0.9	34.7
7	0.6	48.7	0.3	64.7	0.6	40.2	....	....	....	....
8	0.7	43.8	....	....	....	....	....	....	....	....

* The cystic ducts of birds 1 and 2 were cannulated 24 hours prior to the experiment. † The cystic ducts of birds 3, 4, and 5 were cannulated at the time of the experiment; bird 3 served as a control for treated birds 4 and 5.

SUMMARY

A procedure for studying bile secretion by the cockerel is described. The data show that the "true" choleretic action reported for gallogen ® in man and mammalian experimental animals does not manifest itself in the cockerel and, therefore, could not be involved in the "hypocholesterolizing" effect of this compound. Sulfarlem increases the bile volume of the cockerel approximately 200 to 400 per cent.

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Studies on the Histopathology of *Leucocytozoon Simondi* Infection

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In 1890, Danilewsky[1] introduced and used the term Leucocytozoon to designate certain nonpigmented parasites of avian blood which he believed to be inhabitants of the leukocytes.

O'Roke[2-4] worked out the general features of the life history of *Leucocytozoon simondi*, and Huff[5,*] and Fallis *et al.*[6] subsequently contributed to our knowledge of the various stages of its development. Cook[7] has recently resolved the question regarding the type of host cell involved in gametocyte development by demonstrating a complete developmental series in erythroid cells.

Briefly, the life cycle of *L. simondi* is as follows: macro- and microgametocytes are ingested by species of black flies (Simuliidae) as they feed upon infected birds. The zygote which is formed becomes elongated and motile. Resulting oocysts develop to maturity and release sporozoites which are then transmitted by the flies to susceptible birds. These organisms invade cells of the reticuloendothelial system, or epithelial cells of various organs, and develop into schizonts which ultimately mature and produce merozoites. The merozoites, in turn, may penetrate cells of the circulating blood and develop into gametocytes, or they may penetrate other tissue cells and repeat the schizogonous phase.[2-6]

Although a majority of the investigations of Leucocytozoon infection have been concerned with the biology of the parasite, the principal gross lesions in *L. simondi* infection have been reported by O'Roke,[2-4] Huff,[5] Fallis *et al.*,[6] and Chernin and Sadun.[8] Principal pathological alterations in both *Leucocytozoon sakharoffi* infection in crows and *Leucocytozoon smithi* infections in turkeys have been reported by Wingstrand[9,10] and Newberne,[11] respectively.

The purpose of this report is to present a histological description of the various tissue changes induced by *L. simondi*, and to point out certain morphological characteristics of exoerythrocytic stages.

Materials and Methods

Material for study was collected from 42 ducks which had been exposed to the bite of black flies in enzootic areas in the vicinity of the University of Michigan Biological Station. Fifteen of the ducks, shown to be parasitized by the demonstration of gametocytes in Giemsa-stained smears of peripheral blood prepared antemortem, were selected for the following studies on the histopathology.

Seven birds (group 1) were exposed during the summers of 1948 to 1950 by Chernin[12-14] and 8 birds (group 2) were exposed during the summers of 1951 and 1952 by Cook.[7] In both instances, observations were made on the hematology of the parasite. However, various tissues were preserved at necropsy and later utilized in the present study. The ducks in group 1 were heavily infected and died 11 to 16 days following exposure, while those in group 2 were less heavily infected and were destroyed eight to 12 days following exposure. Three unexposed ducks served as controls.

The organs studied were the lungs, liver, kidneys, spleen, brain, heart, intestines, pancreas, and bone marrow. Representative sections from each organ were fixed in Zenker's fluid (9.5:0.5), and were washed, dehydrated, and embedded in paraffin. Sections were cut at 7 μ and stained with hematoxylin and eosin for routine study. Giemsa-stained duplicate sections were prepared from each organ. Serial sections were made from selected organs and the following special stains were employed: Mallory's phosphotungstic acid hematoxylin; Heidenhain's modification of Mallory's stain for connective tissue; Feulgen's stain for thymonucleic acid; Gram's stain; a modification of Perls' Prussian blue reaction for hemosiderin; and Lillie's periodic acid leuco-fuchsin.[15]

The Nature of Lesions Produced by Leucocytozoon Simondi

Host Tissue Reaction to Parasitized Cells.—Both round and elongated forms of gametocytes were observed in the tissues

From a thesis submitted to the Graduate School, Alabama Polytechnic Institute, Auburn, in partial fulfillment of the requirements for the M.S. degree (1954).

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* To preserve continuity, the descriptive terms proposed by Huff are used. He described the hepatic schizont as occurring in and apparently limited in size by hepatic cells, and the megaloschizont as a much larger schizont found in the heart, liver, spleen, and intestine. Cytomere is a term denoting individual formations of masses in the developing schizonts, and the term merozoite denotes individual organisms found in the final stage of schizongony. The stage found in circulating blood is the gametocyte.

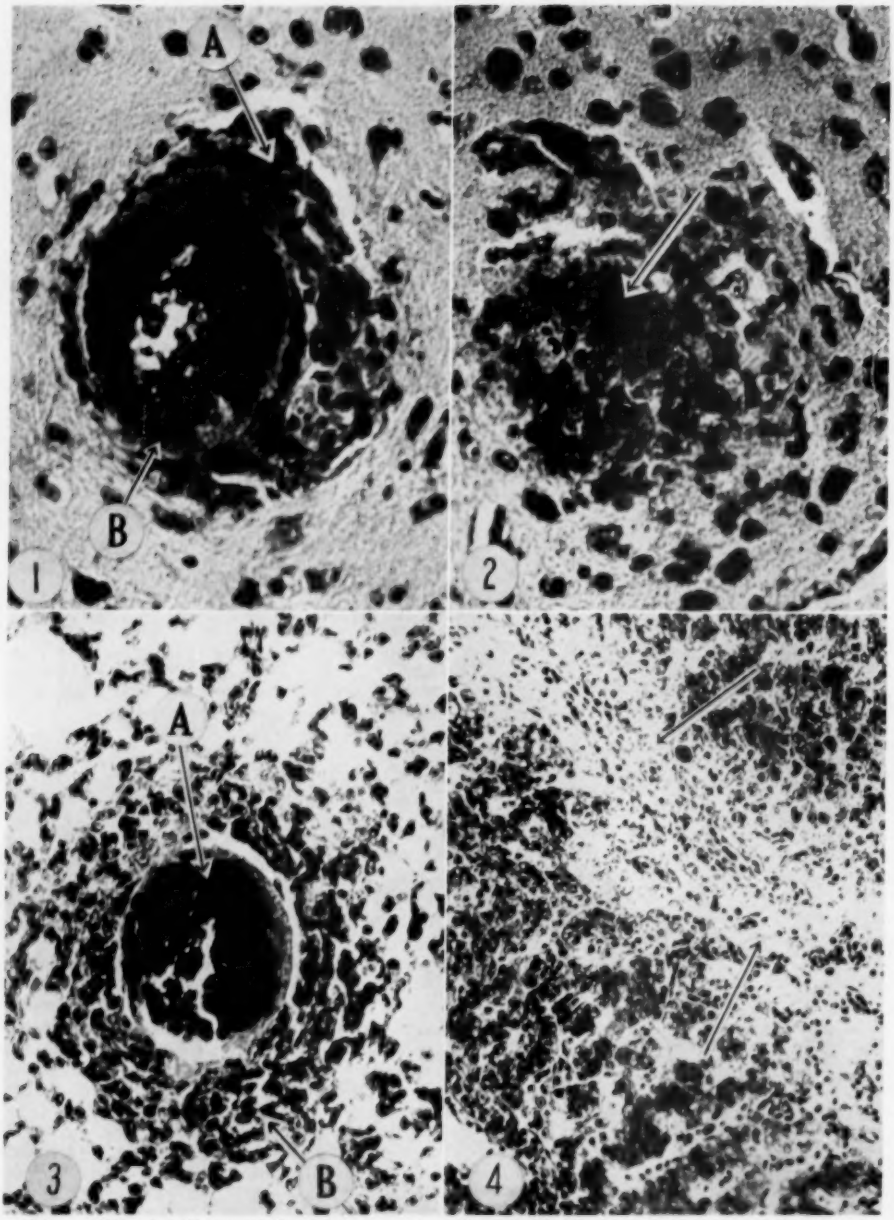

Fig. 1—Megaloschizont of Leucocytozoon simondi in the brain of a duck. Note cellular reaction (A) and round cytomeres (B). Stain, hematoxylin and eosin. x 233.

Fig. 2—Microscopic scar in the brain of a duck, a sequel to rupture of a megaloschizont of L. simondi. Stain, hematoxylin and eosin. x 253.

Fig. 3—Megaloschizont of L. simondi in the lung of a duck (A). Note the prominent cellular reaction (B). Stain, hematoxylin and eosin. x 233.

Fig. 4—Section of liver from a duck with severe L. simondi infection. Note confluent central necrosis (arrows). Stain, hematoxylin and eosin. x 166.

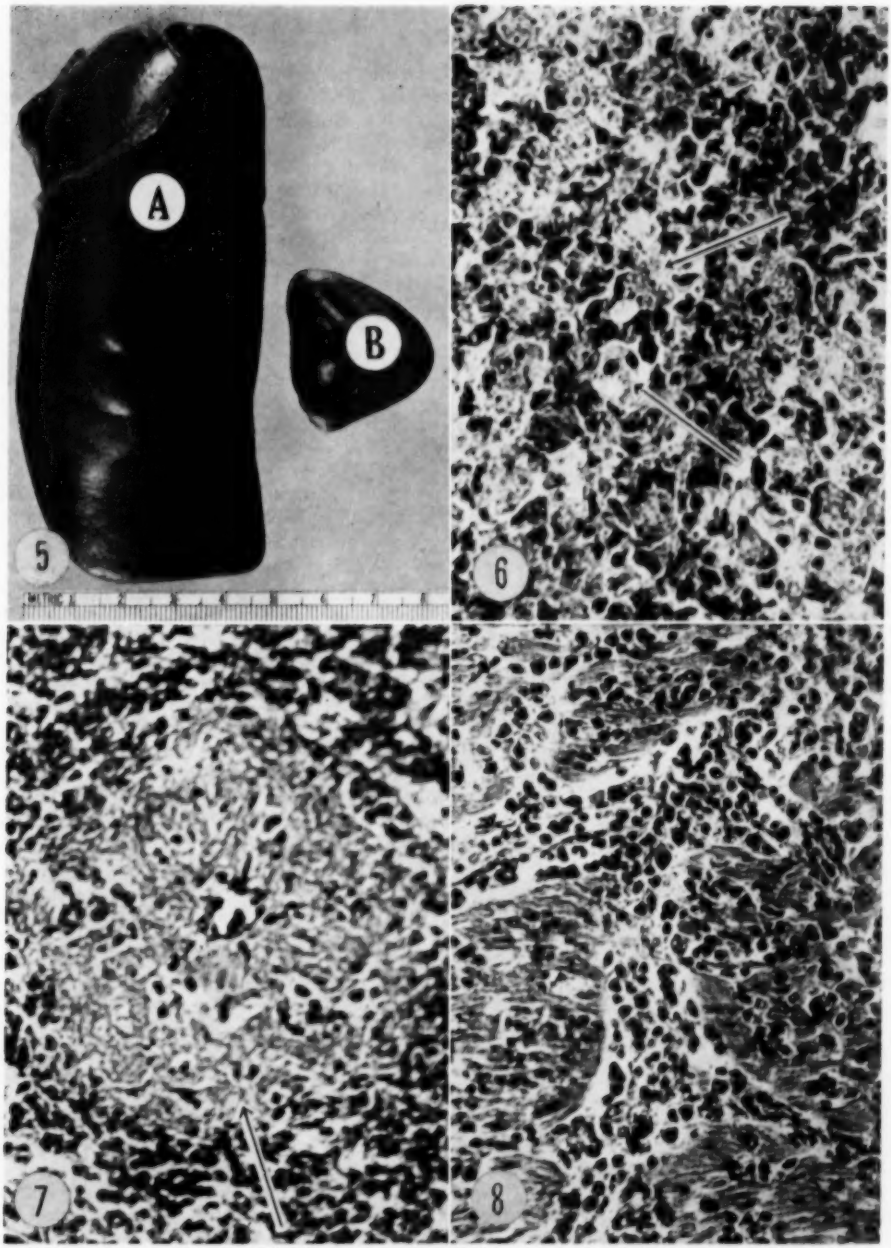

Fig. 5—Gross appearance of spleen from a duck with severe Leucocytozoon simondi infection (A) compared with that of a normal control (B). (Courtesy of Dr. Eli Chernin.)

Fig. 6—Section of spleen from a duck with severe L. simondi infection. Note altered splenic architecture and proliferation of macrophages (arrows). Stain, hematoxylin and eosin. x 300.

Fig. 7—Hyalinization of adenoid sheath cells (arrow) in the spleen of a duck with severe L. simondi infection. Stain, hematoxylin and eosin. x 233.

Fig. 8—Section of cardiac muscle from a duck with severe L. simondi infection. Note diffuse and focal lymphocytic infiltrations. Stain, hematoxylin and eosin. x 233.

of most birds with the lungs, liver, and spleen showing the greatest concentrations. The capillaries of these organs were markedly distended due to the presence of these parasites, but there was no histological evidence of local host tissue reaction to them in any of the infected ducks. Furthermore, in 3 ducks with hepatic schizonts, there was no apparent host tissue reaction to the parasitized hepatic parenchymal cells.

Megaloschizonts were found in the brains of 9 ducks. In three of these brains, there was a moderate to marked cellular reaction around most of the schizonts present (fig. 1), whereas some schizonts in the same sections had elicited little or no reaction. In the remaining six brains, only occasional schizonts were found, and these were associated with slight to moderate cellular reaction. In all cases, the megaloschizonts developed adjacent to, or in close association with, small blood vessels. The host tissue reaction in the brain was characterized by proliferation of large mononuclear cells which apparently stemmed from mesenchymal cells in the perivascular area. An envelope composed of one or more layers of these proliferating cells partially or completely surrounded the schizont. This was invariably thicker on the side nearest the blood vessel. However, in any one section there might have been schizonts about which cellular reaction did not occur. Megaloschizonts which had ruptured and were depleted of merozoites were filled with an eosinophilic coagulum and large mononuclear cells. There were also scattered microscopic scars (fig. 2), apparently the remnants of depleted megaloschizonts.

Megaloschizonts were found in the lung tissue of 3 ducks and elicited a variable host reaction. In 1 duck, a well-marked cellular reaction, several layers thick, was found around the schizont. This consisted of lymphoid cells, plasma cells, and large mononuclear cells interspersed with scattered fibroblasts (fig. 3). Another bird showed a similar but less pronounced reaction, and a third, none.

Megaloschizonts in organs other than the brain and lung failed to elicit any local host tissue reaction.

Phosphotungstic acid hematoxylin and Heidenhain's stains revealed a well-defined wall of reticular fibers which circumscribed megaloschizonts in the liver, spleen, brain, lungs, and kidneys. In contrast, a thin but poorly defined wall of reticular tissue was demonstrated about megaloschizonts in the heart, bone marrow, and intestine.

Pathological Changes Associated with General Systemic Infection.—Liver.—Five birds in group 1 and 1 bird in group 2 showed severe alteration of the liver. There was central necrosis which, in some instances, was so widespread and severe that necrotic areas had become confluent (fig. 4). The damaged areas contained numerous dead parenchymal cells, parasitized blood cells, and macrophages, most of which contained pigment and cellular debris. There were marked periportal lymphocytic infiltration and slight to marked diffuse lymphocytic infiltration throughout the liver. The diffuse lymphocytic infiltration was remarkable in that the infiltrating lymphoid cells were found in both the sinusoids and the hepatic cell cords. Many individual lymphocytes had penetrated the sinusoidal walls and become wedged between hepatic parenchymal cells. Many of these lymphocytes within the liver cords were pyknotic, and their cytoplasm had condensed into an eccentric pink-staining globular mass. Other lymphocytes which had penetrated the sinusoidal walls had not undergone pyknosis and their identity was readily established. Many of the Kupffer's cells in the undamaged areas and macrophages in the necrotic zones were markedly enlarged and contained a variable amount of pigment which gave a positive histochemical reaction for hemosiderin.

Spleen.—All birds in group 1 and 1 in group 2 showed marked enlargement of the spleen (fig. 5). Microscopically, this appeared mainly due to congestion and proliferation of macrophages which resulted in almost complete obliteration of the normal splenic architecture. The typical microscopic field (fig. 6) showed a predominance of blood cells and macrophages and fewer lymphoid and reticular cells. The macrophages were swollen and contained large quantities of pigment and cellular debris. Most of the adenoid sheaths contained considerable hyaline material that separated and displaced the adenoid sheath cells (fig. 7). This hyaline change was most striking in the spleens

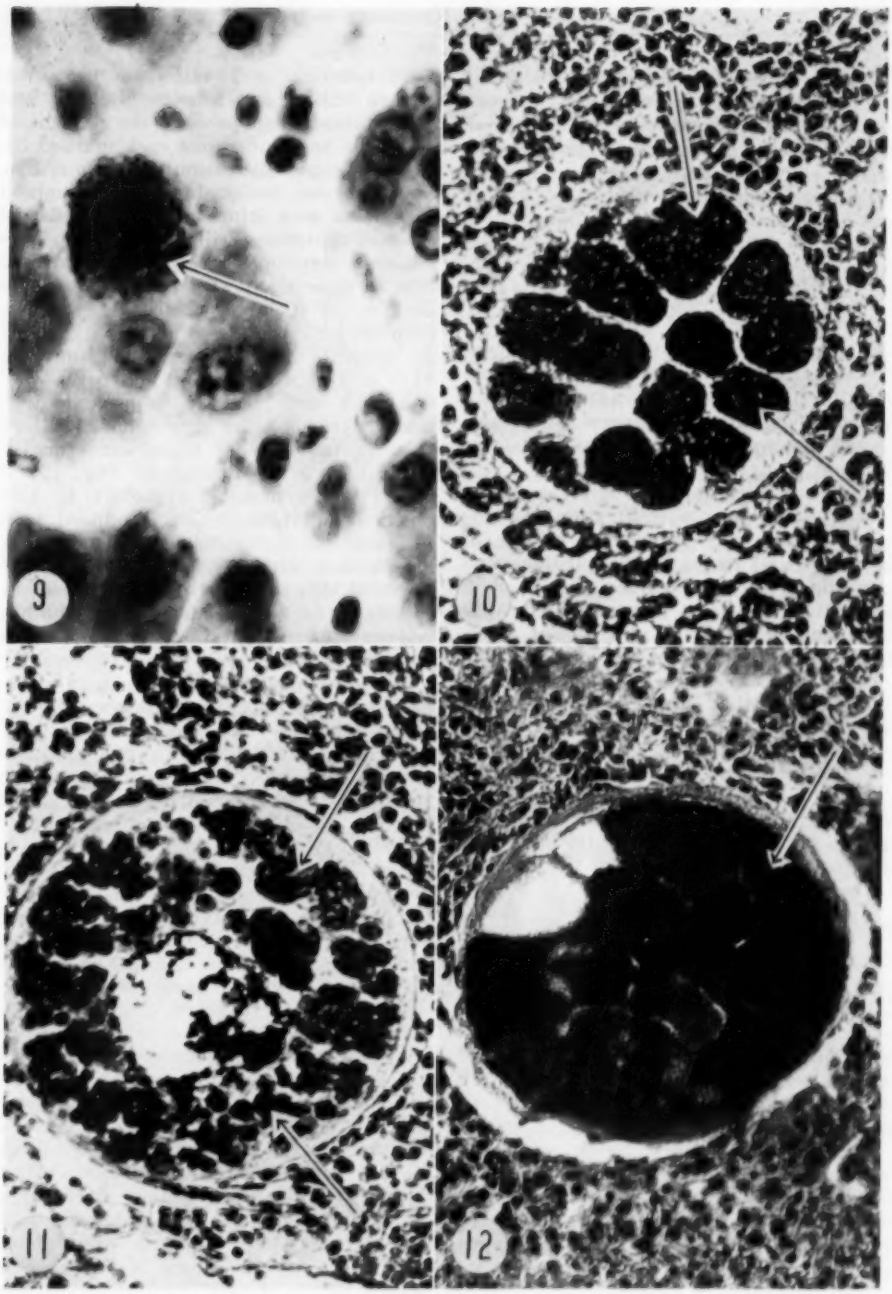

Fig. 9—Hepatic schizont of Leucocytozoon simondi (arrow) in duck liver. Stain, hematoxylin and eosin. x 1,833.

Fig. 10—Megaloschizont of L. simondi in duck spleen. Note island division of cytoplasmic masses (arrows). Stain, hematoxylin and eosin. x 300.

Fig. 11—Megaloschizont of L. simondi in duck spleen. Note that cytoplasmic masses (arrows) are round to ovoid. Stain, hematoxylin and eosin. x 300.

Fig. 12—Megaloschizont of L. simondi in duck spleen (arrows). Note that cytoplasmic masses are approaching homogeneity. Stain, hematoxylin and eosin. x 266.

where the Schweigger-Seidel sheaths could still be recognized. Splenomegaly was not correlated with the presence or absence of megaloschizonts in that organ. In a few instances, the normal architecture remained intact even though the spleens contained large numbers of megaloschizonts.

Lungs.—Several birds from both groups showed pulmonary congestion. The more severely affected were thickened by infiltration of the air space septums with large histiocyte-type cells containing cellular debris and traces of pigment, none of which gave the histochemical reaction for hemosiderin. The lungs of several birds also showed patchy areas of lymphocytic infiltration.

Heart.—Several birds from each group showed lymphocytic infiltration in the heart, primarily around blood vessels of the interstitial tissue in the subepicardial area (fig. 8). Lymphocytes, singly or in aggregates, were also present along capillaries and occasionally in adjacent intermuscular areas deep in the myocardium.

Bone Marrow.—Bone marrow was available from only 5 ducks in group 1. In each case, there was moderate to marked hyperplasia and replacement of fat by actively proliferating cellular elements.

Other Organs.—There were no significant histological changes attributable to general systemic infection in the pancreas, intestines, or brains of any of the infected ducks.

TISSUE STAGES

Tissue stages were found in 12 of the 15 ducks. Hepatic schizonts were found in only 3 birds of group 1. Megaloschizonts were found in all birds of group 1 and in 5 birds of group 2 in one or more of the following organs: liver, spleen, heart, lungs, brain, kidneys, intestines, and bone marrow.

The hepatic schizonts (fig. 9) were similar in essential detail to those described by Huff.[5] They were located within parenchymal cells. Morphological changes of the invaded hepatic cell either did not occur or were very slight, since there was no apparent distortion of either the host cell or its nucleus.

Megaloschizonts in several stages of development were observed in the spleen. The intermediate and more mature forms were spherical and varied in size from 52.5 to 122.5 μ in diameter. In all stages of the maturation process, the parasitized host cells had undergone such marked hypertrophy and distortion that their identity could not be conclusively determined. In spleens with minor alteration and with adenoid structures still intact, the megaloschizonts developed almost without exception in association with Schweigger-Seidel sheaths. A stage in which the cytoplasm divided into islands is shown (fig. 10). In several of the megaloschizonts (fig. 11), a majority of the cytomeres were spherical and usually separated from other cytomeres in the same schizont. Part of the original island structure remained in a megaloschizont (fig. 12) in which the cytomeres appeared to have ripened individually and produced large numbers of merozoites. As the schizont approached ultimate maturity, the pronounced demarcation of islands and cytomeres tended to disappear, and the host cell cytoplasm contained a homogeneous mass of merozoites.

Phosphotungstic acid hematoxylin revealed a differential staining reaction of the cytomeres. The younger cytomeres were markedly acidophilic, but gradually became basophilic as they matured and produced merozoites. Several schizonts contained both young acidophilic cytomeres and more mature basophilic cytomeres and merozoites, indicating asynchronicity. Demonstration of chromatin by Fuelgen's stain for thymonucleic acid was not possible since these tissues had been fixed in Zenker's fluid. The internal structures of the schizonts did not stain differentially with Gram's stain, periodic acid leuco-fuchsin stain, or Heidenhain's modification of Mallory's connective tissue stain.

Megaloschizonts in the brain differed in morphological development from those in the spleen. They, too, were spherical and showed greater variation in size (14.0 to 117.5 μ), but early island divisions, so prominent in the spleen, were not seen. The earliest stage observed, even though small, already contained spherical cytomeres which showed considerable variation in size (fig. 1).

Megaloschizonts in the heart varied considerably in morphology from those in both the spleen and brain. They generally de-

veloped in capillaries or adjacent tissue, and were elongate in longitudinal section (fig. 13) and ovoid in cross section (fig. 14).

Megaloschizont development in the kidneys, intestines, lungs, and bone marrow was similar in general detail to that in the spleen.

Discussion

Local host tissue reaction to megaloschizonts occurred in the brain and lungs but not in other organs, and not to hepatic schizonts or gametocytes. The paucity of tissue reaction is not surprising, since the parasite is intracellular. Because all stages of the parasite are generally assumed to be intracellular, a logical explanation for the tissue reaction which occurred in relation to megaloschizonts in the brain, and to some of those in the lungs, was not determined.

Megaloschizonts in all the organs were enveloped by a capsular wall of reticular fibers. This reticular capsule is not properly classified as a tissue reaction *per se*, except in the brain, since it apparently results from condensation of pre-existing reticular fibers on the surface of the expanding megaloschizont. In the brain, however, the megaloschizonts develop within cells directly associated with blood vessels, rather than in the nervous tissue. Since the nerve tissue as such does not contain reticulum, the reticular wall that develops around the schizont apparently develops from the proliferation of large, mononuclear, reticulum-producing mesenchymal cells associated with the blood vessels. The cellular reaction around any given schizont was heaviest on the side nearest the associated blood vessel, since it was from the vicinity of this vessel that the reactive cells were derived. Thus, the intensity of cellular reaction seen around a given megaloschizont in the brain varied with the level at which the parasite was sectioned.

Histopathological alterations attributable to general systemic infection in the birds of the two groups showed a close correlation with the clinical severity and duration of the infection. With some vari-

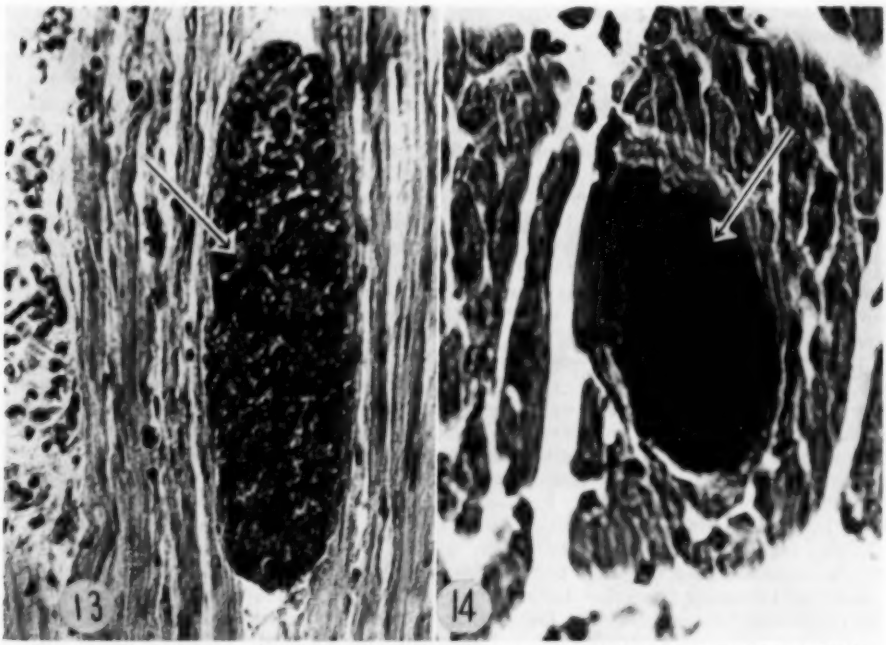

Fig. 13—Elongated megaloschizont of L. simondi (arrow) in longitudinal section of duck cardiac muscle. Stain, hematoxylin and eosin. x 300.

Fig. 14—Ovoid megaloschizont of L. simondi (arrow) in cross section of duck cardiac muscle. Stain, hematoxylin and eosin. x 466.

ation among individuals, all those that died from acute fulminating infection (group 1) showed well-marked liver damage and enlargement of the spleen from congestion and macrophage proliferation. In addition, there were periportal and diffuse lymphocytic infiltration in the liver, and moderate to marked hyalinization of the adenoid sheaths in the spleen. The principal alterations in the liver and spleen can be attributed to massive red blood cell destruction and resultant anemia. This point is of particular interest since Cook [7] has shown the red blood cell to be the host cell of the mature gametocyte, and Fallis [6] has recorded extremely low hemoglobin and erythrocyte determinations in infected birds. Anoxia from anemia appears to be the underlying cause of the liver damage. However, the enlargement of the spleen from congestion and macrophage proliferation is a reaction to the large amount of erythrocyte destruction incident to the infection. Thus, fatal termination in Leucocytozoon infection may be attributed to severe anemia, to the pathological alterations in the liver resulting therefrom, and to respiratory embarrassment due to the presence of large numbers of gametocytes in the capillaries of the lungs.

The lymphocytic infiltration seen in several ducks is not considered pathognomonic of leucocytozoonosis, but rather a general reaction of the lymphoid tissue to the disease process. In this connection, it should be mentioned that because of the peculiar staining affinity that lymphocytes acquire when they infiltrate the liver cords and undergo degeneration, they may be difficult to distinguish from very early schizogenous stages of the parasite.

In retrospect, it seems significant that the pathological changes observed in the two groups of birds showed characteristic variations according to the severity and duration of the infection. This knowledge should aid both the research worker and the diagnostician in evaluating Leucocytozoon infections generally. Further, it emphasizes the necessity for more than a simple demonstration of gametocytes in determining mortality from this disease, since the birds could conceivably die from other causes, yet concomitantly harbor a light, inapparent Leucocytozoon infection.

Hepatic schizonts were demonstrated in 3 ducks only after a thorough search of the material. The parasite develops within hepatic parenchymal cells. There is no apparent significant morphological difference between hepatic schizonts of *L. simondi* and those of *L. smithi* of turkeys, described in a previous paper.[11] The minimal morphological alteration of the invaded hepatic cells is in contrast to the effect of the megaloschizont on its host cell. Huff,[5] in attempting to mediate the relationship between hepatic schizonts and megaloschizonts of *L. simondi*, tentatively presented three hypotheses: (1) The two kinds of schizonts may be different stages in the same life cycle; (2) they may be essentially similar stages except for location, with those entering liver cells being limited in size by the type of host cell; or (3) they may represent stages in the life cycles of two different species of parasite. Consideration of these hypotheses becomes even more interesting in view of the recent demonstration of a hepatic stage in *L. smithi* infection [11] in which only elongated forms of gametocytes were observed. Previously, both hepatic and megaloschizonts have been observed in *L. simondi* infection, and both elongated and round forms of gametocytes occur. The present study, however, does not aid in resolving the problem of which hypothesis may be more nearly correct.

In general, stages in the development of megaloschizonts of *L. simondi* observed in the spleen, in this study, were similar to those described by Huff.[5] However, the megaloschizonts observed in the heart appeared to develop only in cells in the intermuscular areas, which does not support or disprove Huff's tentative suggestion that megaloschizonts may actually develop in cardiac cells as well. Their elongate shape is probably attributable to the natural compression exerted by the muscle tissue which surrounds the megaloschizonts as they develop.

Variations in development of megaloschizonts in the brain, as compared with those in the spleen and other organs, can not be explained at present. A logical reason for the consistent absence of large island structures and the early presence of round cytomeres in those in the brain was not determined. Cowan [10] considered the brain to be an unfavorable site for the parasite's development. This view is diffi-

cult to reconcile with observations made in this study. Megaloschizonts measuring 117.7 μ in diameter, a size which exceeds some of the mature forms seen in the spleen, were found, and numerous depleted megaloschizonts and microscopic scars resulting therefrom were seen, indicating that these stages had matured and ruptured.

The general observation may be made that there was no one organ in which megaloschizonts were most likely to be found in any given bird. As suggested by Huff,[6] this may explain the difficulty some investigators have experienced in finding megaloschizonts when only certain organs were examined.

SUMMARY

1) Studies were made of the histopathological changes in two groups of ducks infected with *Leucocytozoon simondi*.

2) Significant lesions found in ducks with severe, fatal infection were: liver necrosis; marked enlargement of the spleen from congestion and macrophage proliferation; periportal and diffuse lymphocytic infiltrations in the liver; moderate hemosiderosis in both the liver and spleen; and hyalinization of the splenic adenoid sheaths.

3) Ducks with only moderately acute infection showed histopathological alterations similar to, but less severe than, those seen in ducks with fatal infections. Those with light, nonfatal infections showed no evidence of a general systemic reaction.

4) Local host tissue reaction was seen to occur only in relation to megaloschizonts in the brain and lung tissue, but not to those in other organs, nor in relation to hepatic schizonts or gametocytes.

5) Megaloschizonts, regardless of location, were enveloped by a capsular wall of reticular fibers which, in all organs except the brain, developed from condensation of pre-existing reticular fibers on the surface of the expanding megaloschizont.

6) Megaloschizonts and hepatic schizonts observed are briefly described, and the essential morphological differences between megaloschizonts occurring in different organs are reported.

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Infectious Synovitis Control. II. A Comparison of Levels of Antibiotics

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INFECTIOUS SYNOVITIS has been described in chickens [1-4] and turkeys.[5] In a previous paper, chlortetracycline, oxytetracycline, and erythromycin were used in the feed at the rate of 200 Gm. per ton of feed to control the disease.[6] Complete control was obtained when either chlortetracycline or oxytetracycline were given continuously after the birds were 1 day old. Erythromycin was not effective. These birds were inoculated in the foot pad at 23 days of age and were then observed for three to seven weeks. Wills and Delaplane [7] found chlortetracycline more effective than oxytetracycline for the treatment of synovitis. Dosages of 200 to 500 Gm. per ton were used for periods of five to 15 days. Withdrawal of medication resulted in recurrence of the infection in some birds.

To determine the efficacy of various levels of antibiotics in the feed for the control of infectious synovitis, chlortetracycline,* oxytetracycline,† streptomycin,‡ and penicillin § were incorporated in a commercial broiler ration so as to give 50 to 400 Gm. of the active drug per ton of feed.

MATERIALS AND METHODS

Starting with 50 Gm. per ton of feed, the level of chlortetracycline, oxytetracycline, and the equal mixture of these two was increased by 25 Gm. increments up to 200 Gm. per ton, with a maximum of 250 Gm. per ton included. Streptomycin, penicillin, and equal parts of the two were fed at 100 and 200 Gm. per ton. Streptomycin was also fed at 400 Gm. per ton. These medicated feeds were given continuously from 48 hours before inoculation to the end of the 18-day experimental period. The birds were in 32 batteries in an air-conditioned room. When 4 weeks old, 12 chicks nearest the mean weight were randomly selected for each battery. The chicks were unsexed and no attempt was made to include equal numbers of pullets and cockerels in each battery.

At 5 weeks of age, 10 chicks in each battery were inoculated in the foot pad with 0.25 ml. of a yolk culture of the infectious synovitis agent harvested from the third embryo passage. The yolks were pooled and diluted 25 per cent with nutrient broth to facilitate handling. Part of the yolk (0.25 ml.) was injected into the foot pad of 5 chickens to test its pathogenicity; the remainder was held at −20 C. until needed. On the day of inoculation, the yolk material was thawed and held in an ice-water bath until inoculations were completed. As controls, 2 chicks in each battery were not inoculated and one pen of inoculated birds was given no antibiotics.

Following inoculations, the birds were observed daily. Every three or four days they were weighed and the development of swellings in the foot pad or other joints was recorded. Necropsies were performed on all birds 17 or 18 days following inoculation and their degree of infection was classified. The inoculated foot pad of 1 bird from each battery was opened and either fluid or scrapings were placed on nutrient agar and in thioglycollate medium to check for contamination. When an exudate was present in the foot pad or other joints, 0.25 ml. of a broth suspension of this exudate was inoculated into the yolk sac of embryonating chicken eggs in an attempt to recover the infectious agent.

RESULTS

The weight gains during a 17-day observation period are given (table 1). Since the sexes were not equally distributed in these pens and the number of birds involved was small, the weight gains should be used only as an indicator of response to the drugs. The number of birds showing lesions (table 2) and the degree of infection (table 3) must also be considered in evaluating the efficacy of the drugs involved. The weight gains (table 1) and

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* Supplied as aureomycin® T.F.-15, each pound of which contained 15 Gm. of chlortetracycline, by Lederle Laboratories, Pearl River, N. Y.

† Supplied as terramycin® TM-10, each pound of which contained 10 Gm. of oxytetracycline, by Chas. Pfizer and Co., Terre Haute, Ind.

‡ Supplied as streptomycin premix, each pound of which contained 50 Gm. of streptomycin, by Merck and Co., Rahway, N. J.

§ Supplied as pro pen 50, each gram of which contained 50 per cent procaine penicillin, by Merck and Co.

TABLE 1—Weight Gain or Loss for a 17-Day Period Following Inoculation * with Infectious Synovitis Agent

Drug level (Gm./ton)	Chlortetracycline	Oxytetracycline	½ chlortetracycline, ½ oxytetracycline	Streptomycin	Penicillin	½ streptomycin, ½ penicillin
50	288	277	190	.....	.....	.....
75	335	267	318	.....	.....	.....
100	346	321	314	180	2	82
125	357	351	390	.....	.....	.....
150	390	363	363	.....	.....	.....
175	343	387	392	.....	.....	.....
200	370	369	369	141	−25	39
250	353	436	446	.....	.....	.....
400	.....	.....	.....	65	.....	.....
None	195					
Contact controls	382					

* Ten inoculated and 2 contact control birds per pen. Weights expressed in grams.

degree of infection (table 3) indicated therapeutic activity of chlortetracycline, oxytetracycline, and equal parts of the drugs against the infectious synovitis agent at the 50-Gm. per ton of feed level. The relatively poor growth response of the birds on the 50-Gm. level of equal parts of chlortetracycline and oxytetracycline and the 250-Gm. level of chlortetracycline was probably due to the sex distribution. There was an increase in weight gain as the level of the tetracycline antibiotics was increased. Generally, there were no marked differences between the weight gains of the birds on chlortetracycline, oxytetracycline, or a combination of the two. All birds on streptomycin, penicillin, and equal parts of these drugs gained less than the untreated inoculated birds. No signs or lesions of infectious synovitis developed in the contact controls.

The number of birds which developed visible lesions is shown (table 2). In the chlortetracycline group, the number of birds showing swelling in the foot pads gradually decreased as the drug level increased from 75 to 200 Gm.; at the latter level, no birds had swellings. At the 250-Gm. level, however, 1 bird had a slight swelling on the seventeenth day after inoculation. The same general improvement occurred with the combination of chlortetracycline and oxytetracycline, except the reduction of lesions did not begin until a level of 125 Gm. per ton was fed. Oxytetracycline was less effective than chlortetracycline in controlling the development of swellings; even at the 250-Gm. level, 8 of 10 birds developed swellings. There was a gradual increase in the number of birds showing swellings four to 17 days following inoculation when the higher levels of tetracycline antibiotics were given. The degree of infection in these birds was slight. These results indicated that the antibiotics prolonged the incubation period and that the agent was not killed. Streptomycin, penicillin, and a combination of

TABLE 2—Number of Birds Having Swellings in the Foot Pad * Inoculated with Infectious Synovitis Agent

Drug level (Gm./ton)	Days after inoculation				
	4	7	10	14	17
Chlortetracycline					
50	7	10	10	10	10
75	2	7	8	9	9
100	1	6	8	8	8
125	1	5	4	6	5
150	1	2	3	4	4
175	0	3	2	3	3
200	1	1	0	0	0
250	0	0	0	1	1
Oxytetracycline					
50	10	10	10	10	10
75	9	10	10	10	10
100	9	10	10	10	10
125	10	10	10	9	9
150	6	9	9	9	9
175	8	8	8	9	8
200	3	7	7	6	6
250	2	6	6	8	8
Equal parts chlortetracycline and oxytetracycline					
50	10	10	10	10	10
75	9	9	10	10	10
100	10	10	10	10	10
125	4	6	7	9	9
150	2	4	2	4	6
175	2	1	2	5	4
200	3	3	3	2	3
250	0	0	0	1	1
Streptomycin					
100	10	10	10	10	10
200	10	10	10	10	10
400	10	10	10	10	10
Penicillin					
100	9	10	10	10	10
200	10	10	10	10	10
Equal parts streptomycin and penicillin					
100	10	10	10	10	10
200	9	10	10	10	10
None	10	10	10	10	10
None (contact controls)	0	0	0	0	0

* Ten inoculated and 2 contact controls birds per pen.

these two drugs had no effect on the course of the disease.

The birds which did not die were killed 17 or 18 days following inoculation and, on necropsy, the degree of joint involvement was classified (table 3).

As the level of chlortetracycline, oxytetracycline, and equal parts of these drugs was increased, the lesions became less extensive. No lesions occurred that were more severe than the "2" classification at, or above, the 125-Gm. level of chlortetracycline, oxytetracycline, and the two combined. At this level and above, the lesions were generally localized. This may account for the lack of differences in weight gains, even though the chlortetracycline was more effective in preventing the development of swelling than were oxytetracycline and equal parts of the two. There were no marked differences between those birds receiving no medication and those receiving the streptomycin, penicillin, and equal parts of streptomycin and penicillin.

TABLE 3—Classification of Lesions at Necropsy of Chickens Inoculated with Infectious Synovitis Agent

Drug level (Gm./ton)	Normal	Classification* 1	2	3	4	5	Deaths
Chlortetracycline							
50	0	0	3	4	3	0	0
75	2	0	7	0	1	0	0
100	0	2	6	2	0	0	0
125	3	1	6	0	0	0	0
150	7	2	1	0	0	0	0
175	6	2	2	0	0	0	0
200	10	0	0	0	0	0	0
250	9	0	1	0	0	0	0
Oxytetracycline							
50	0	0	7	1	2	0	0
75	0	1	5	1	3	0	0
100	0	0	9	1	0	0	0
125	0	0	9	0	1	0	0
150	0	1	9	0	0	0	0
175	2	3	5	0	0	0	0
200	5	2	3	0	0	0	0
250	1	4	5	0	0	0	0
Equal parts chlortetracycline and oxytetracycline							
50	0	0	5	1	4	0	0
75	0	0	7	3	0	0	0
100	0	1	6	1	2	0	0
125	1	3	6	0	0	0	0
150	6	2	1	0	0	0	0
175	3	6	1	0	0	0	0
200	5	3	2	0	0	0	0
250	7	1	2	0	0	0	0
Streptomycin							
100	0	0	2	4	2	2	0
200	0	0	1	3	5	1	0
400	0	0	0	2	1	6	1
Penicillin							
100	0	0	0	1	2	7	0
200	0	0	2	1	1	4	2
Equal parts streptomycin and penicillin							
100	0	0	0	2	3	3	2
200	0	0	0	0	8	2	0
None†	0	0	0	2	3	1	1
None (contact controls)	64	0	0	0	0	0	0

* Classification: 1—slight thickening of pad or tendon sheath; 2—thickening of pad or tendon sheaths; 3—marked thickening of pad, usually fluid and caseous exudate; 4—same as 3 with extension of infection to other joints; and 5—generalized infection.

† Three birds held for further study were markedly infected.

Recoveries of the infective agent in egg embryos inoculated with synovial fluid from infected birds were attempted from 16 birds and were successful in 10. The agent was recovered from the birds given the following levels of antibiotics expressed in grams per ton of feed: chlortetracycline, 100 and 125; oxytetracycline, 75, 100, 125, and 150; streptomycin, 200; penicillin, 100 and 200; and equal parts of streptomycin and penicillin, 100. The agent was not recovered from birds given the following (Gm./ton of feed): chlortetracycline, 50; oxytetracycline, 50; equal parts of chlortetracycline and oxytetracycline, 50 and 75; streptomycin, 100; and equal parts of streptomycin and penicillin, 200. When a suspension of synovial fluid was placed on nutrient agar or in thioglycollate medium, no growth occurred.

DISCUSSION

The results of these tests indicate that the optimum level of chlortetracycline in the feed for the control of synovitis is about 200 Gm. per ton. For oxytetracycline, the level would be somewhat higher. Localization of the lesions occurred with levels of 125 to 150 Gm. and above for chlortetracycline, oxytetracycline, and equal parts of these drugs. Generally, the chlortetracycline and a combination of chlortetracycline and oxytetracycline were more effective than oxytetracycline in preventing the development of lesions, but no marked differences occurred in weight gains.

The period of this experiment was only 18 days. Since there was a tendency for some birds to develop slight swellings as long as 17 days after inoculation, a longer experimental period may have resulted in more birds developing swellings. The age of the bird should also be taken into consideration since Shelton *et al.*[8] reported a greater efficacy of chlortetracycline in 2-week-old birds. The reverse may also be true in that higher levels of the antibiotics may be required to control infectious synovitis in birds over 5 weeks of age. Differ-

ent results might have been obtained with other strains of the agent. Also, the pathogenicity of cultures may vary.

The presence of swellings in birds (table 2) was not always confirmed by necropsy (table 3). The differences in these observations was minor and occurred only in those birds with slight swellings.

These birds were probably given a greater exposure than birds would receive under field conditions. This may account for the favorable reports from the field indicating that levels of 100 Gm. of chlortetracycline per ton used continuously from day-old controlled infectious synovitis.

SUMMARY

Under the conditions of this experiment, there were no marked differences in weight gains in birds on equivalent levels of chlortetracycline, oxytetracycline, and equal parts of the two. Chlortetracycline was more effective in controlling the development of swellings than were either oxytetracycline or a combination of oxytetracycline and chlortetracycline. Streptomycin and penicillin given in the feed were not effective in controlling infectious synovitis.

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Further Studies with the Aqueous Humor of Chickens as a Reservoir for the Virus of Newcastle Disease

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IN 1954, McClurkin *et al.*[1] reported on a rapid test for early detection of Newcastle disease. Their test made use of hemagglutination (HA) reaction, using fluid extracted from lung tissues which had been frozen. Clark *et al.*,[2] in 1955, observed that aqueous humor compared well with lung tissue as a source of the virus. These reports indicated that the test was of value chiefly for the detection of the disease in its incipient stages, before blood serum would produce positive hemagglutination-inhibition (HI) reaction. The latter authors observed that low titer reactions needed further investigation and recommended that they be used guardedly in making a diagnosis.

The test using aqueous humor has been used in the San Gabriel laboratory for approximately two years. It has been employed on more than 3,000 birds, representing 1,300 groups of chickens, which were submitted for diagnosis. Parallel HI tests were performed with more than 90 per cent of these birds.

Early experience with the test revealed that a high percentage of positive reactions were occurring. Most of the positive HA reactions could not be substantiated by HI tests, and there were rarely nervous signs or lesions suggestive of Newcastle disease. Furthermore, usually vaccination against this disease appeared to be adequate.

During the past year, a special study of the HA test has been made in an effort to determine the significance of such frequent occurrences of positive reactions. As a part of this study, the test has been used, more or less routinely, not only when Newcastle disease was suspected but also when other conditions, such as bluecomb disease, coccidiosis, lymphomatosis, and reproductive disorders, were primarily involved.

Groups of chickens from 995 flocks, submitted to the laboratory for diagnosis from June, 1955, to May, 1956, inclusive, were used in this study. This excluded those in which an HA reaction might be attributed to recent live virus vaccination. An average of 2.3 birds per group were individually tested by the HA and HI methods. A group was designated as HA-positive when aqueous humor of one third or more of the individuals in the group tested produced an HA reaction in a dilution of 1:40 or higher. Groups positive to the HI test were so recorded if the serum of at least one third of the birds in a group gave a positive reaction in a dilution of 1:20 or higher. Of the 995 groups thus classified, 70 per cent proved to be HA-positive and only 31 per cent were HI-positive.

To facilitate further investigation, the groups were segregated into two age categories. Those involving birds 5 months old or more were designated as adults and those less than 5 months old as growing birds.

The adults consisted of 299 groups. Of these, 83 per cent were classified as HA-positive. Included were 135 groups of normal-appearing adult pullets submitted for laboratory diagnosis with a history of a reproductive problem. Common complaints were a delay in beginning production, the cessation of production after starting, a continuous low flock production, or no production at all. Of these groups with a reproductive disorder, 90 per cent proved to be HA-positive. In 143 groups not involving reproductive disorders or frank Newcastle disease, 68 per cent were found to be HA-positive. The remaining 21 groups of adults were diagnosed as frank Newcastle disease.

There were 696 groups less than 5 months old, 64 per cent of which were classified as HA-positive. A chronic respiratory infection involving primarily the air sacs was the principal problem in 382 groups in this age category, 67 per cent of which were HA-positive. In 153 groups in which there was no evidence of a chronic respiratory infection or frank Newcastle

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disease, 53 per cent proved to be HA-positive. The remaining 161 groups of growing birds were diagnosed as frank Newcastle disease.

It was of considerable interest that of 38 groups of nonvaccinated birds, 2 weeks old or less, 45 per cent were HA-positive. In one instance, apparently normal chicks, 2 days old, produced an HA reaction, and a lethal Newcastle disease virus was isolated from the aqueous humor.

TABLE 1—Vaccination History in Relation to Hemagglutination (HA) Reactions in Birds 5 Months or More of Age

Vaccine used	No. of groups	HA-positive (No.)	HA-positive (%)
None	65	56	86
Killed virus only	52	42	81
B1 type	100	81	81
Wing-web type	82	68	83
Totals	299	247	83

A study of the Newcastle disease vaccination status of adult flocks showed no significant difference in HA reactions among nonvaccinated flocks and those using various types of vaccines. The results of this study are shown (table 1). With the growing birds, accurate correlations were impossible because of the variation in ages and stages in the vaccination program.

The high incidence of positive HA reactions suggested the need for a more critical study to determine their specificity for the virus of Newcastle disease. A project was developed to study the virus contained in aqueous humor of such birds.

MATERIAL AND METHODS

Fluid from the anterior chamber of the eyes was found to be a better source of the virus than the fluid from the posterior chamber. The material is obtained by using an ordinary glass syringe and a 19- to 20-gauge needle. With the bevel of the needle down, the point is inserted into the lateral aspect of the anterior portion of the eye, thus entering the anterior chamber. With a steady suction, the fluid is drawn into the syringe. Material from both eyes is pooled and instilled into a small glass tube, which is corked and identified by case number and bird number. As a rule, two or three specimens were obtained from each accession. From the same birds, blood was obtained by cardiac puncture, identified in a similar manner, and slanted to separate serum for HI tests.

Specimens of aqueous humor were frozen at —10 C. until several were accumulated for HA tests. Those producing HA reactions in dilutions of 1:20 or higher were frozen until embryos of a proper age were available for inoculation. Only aqueous humor from groups in which all individuals gave negative HI tests was used. Special effort was made to select material only from groups in which symptoms and history were not suggestive for frank Newcastle disease and in which the reaction could not be attributed to recent vaccination with a live virus.

Embryo Inoculation.—Prior to inoculation, the eye fluid was treated with a combination of antibiotics containing 10,000 units of penicillin and 100 mg. of streptomycin per milliliter. A volume of this mixture of antibiotic equal to that of the aqueous humor was added and maintained at room temperature for 15 minutes before inoculation of embryos.

Eleven-day White Leghorn chicken embryos were inoculated with 0.2 ml. of the antibiotic and aqueous humor combination via the allantoic route. The eggs were candled morning and evening. Allantoic fluid from dead embryos was harvested, titered for hemagglutination, and frozen. Embryos not dead after seven days were discarded. No second passages were attempted.

To confirm that the virus isolated was Newcastle disease virus, the extract hemagglutination test, using specific anti-Newcastle disease serum, was performed with each virus isolated.

Chick Inoculations.—Nonimmune chicks, 4 weeks old, were inoculated intramuscularly with 0.2 ml. of a 10^{-4} dilution of HA-positive allantoic fluid from dead embryos. Three chicks were inoculated with each virus representing a field case. Inoculated birds were observed twice daily and the time of onset and physical signs displayed were recorded.

RESULTS AND DISCUSSION

Virus isolations were attempted with aqueous humor from 127 individual birds, each representing a different group. Adults were represented in 54 instances and growing birds in 73 (table 2). There were 111 first-passage isolations made. These represented individual birds from 46 groups of adults and 65 of growing birds, six of which were from nonvaccinated chicks less than 2 weeks old.

It will be noted that 95 per cent of the aqueous humor samples having a titer of

TABLE 2—Results of Single-Passage, Chicken Embryo Virus Isolation Trials, Using Aqueous Humor with Various Hemagglutination (HA) Titers

HA titer of aqueous humor	Isolations attempted	Isolations (No.)	Isolations (%)
1:80 or above	41	39	95
1:40	55	47	85
1:20	31	25	81
Totals	127	111	87(av.)

1:80 or higher and 85 per cent of those with a titer of 1:40 yielded Newcastle disease virus. It is significant that the virus was recovered from 81 per cent of the samples with a titer of 1:20.

Using the extract hemagglutination test, specific anti-Newcastle disease serum was found to inhibit hemagglutination with all viruses isolated.

Viruses producing embryo deaths in first-passage trials showed a similar death pattern. All mortality occurred within 72 hours, with most deaths occurring during the first 48 hours.

A similar pattern was also seen in the chick inoculation trials. All viruses isolated proved to be lethal to susceptible chicks. In each instance, nervous signs, typical of Newcastle disease, occurred three to five days following intramuscular inoculation.

Early embryo mortality and the high virulence to test chicks suggest that a rather lethal field strain of Newcastle disease virus was usually involved.

Late in the experiment, spot trials revealed that allantoic fluid from many inoculated embryos not dying on first passage produced hemagglutination. This indicated that if further embryo passages had been attempted, it might have been possible to make more isolations, including some of the less lethal strains of Newcastle disease virus.

These results help to confirm the specificity of positive HA reactions for the virus of Newcastle disease. The frequent occurrence of Newcastle disease virus in aqueous humor of chickens and its relation to other diseases, particularly those of reproduction in adults and chronic respiratory infections in growing birds, warrants further investigation.

SUMMARY

Further studies with the hemagglutination (HA) test for Newcastle disease, using aqueous humor of chickens, are reported. The test, used on 995 routine diagnostic accessions of poultry, produced positive HA reactions in 83 per cent of adult groups of birds and in 64 per cent of the groups involving growing birds. The majority of HA-positive reactions could not be substantiated by hemagglutination-inhibition (HI) tests, and nervous signs characteristic of frank Newcastle disease were usually absent.

Disease conditions most often associated with positive reactions were reproductive disorders in adults and chronic respiratory infections in growing birds. Many positive reactions also occurred with other disease conditions.

Virus isolation studies, using aqueous humor from 127 HA-positive birds, resulted in isolation of a lethal strain of Newcastle disease virus in 111 instances (87%). It was concluded that the aqueous humor is a reservoir for the virus of Newcastle disease in a large percentage of chickens in southern California.

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The Occurrence of Certain Enterobacteriaceae in Birds

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WHILE the distribution of most Enterobacteriaceae in domestic fowl and in caged birds is well known, to the knowledge of these writers no extensive survey of enteric bacteria in wild birds has been carried out. The question of the relationship of man, animals, and birds to vegetables and soil and to the microbial contamination of food has been the subject of many inquiries. This problem is being investigated in this laboratory in an effort to measure the extent of fecal contamination of vegetables grown in the Far East. This report deals with the examination of the possible role of several species of wild birds in the distribution of enteric bacteria which are pathogenic to man. Many species of enteric bacteria found in man are harbored and excreted by other mammals and by birds.

MATERIALS AND METHODS

Sixty-one fecal specimens were collected from 13 species of birds. The bulk of these came from ardeids in colonies situated as follows: At Tokyo, one was in trees and bamboo within and surrounding five farmyards in an upland habitat above narrow valleys devoted to rice cultivation, and one was at a duck refuge on the shore of Tokyo Bay in dense bamboo thickets surrounded by rice paddies. At Nagoya, one was in low pine trees in an upland habitat adjoining a small lake, and one was in a pine- and cedar-forested gully in low mountains away from cultivation.

The Nagoya site by the lake was also used by a large colony of cormorants. All cormorant specimens came from this colony. Fecal material of the ardeids and cormorants was collected as it was voided, while the nestlings were being handled for banding and other studies.

Thirteen fecal samples were taken from nestling Japanese blue magpies in an upland habitat northwest of Tokyo. These nests were in shade trees at farmyards or in second growth deciduous forests. Two samples came from streaked shearwater adults taken from nest burrows on an uninhabited island off the east coast of northern Honshu. Additional samples were from species which had been in an outdoor aviary in Tokyo for periods of a year or more.

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It is not within the scope of this paper to discuss diets in detail, but briefly the diets of the black-crowned night heron, great egret, plumed egret, and snowy egret are predominately fish, frogs, and crustaceans found about rice paddies and in the tide flats. The proportion of each food taken varies considerably for each species. The diet of the cormorant consists mainly of salt-water fish caught in Tokyo Bay. The diet of the streaked shearwater includes salt-water fish collected many miles at sea. The blue magpie is a general feeder, preferring insects and fruit.

In the aviary, the diet of the bobwhite, emerald dove, zebra dove, turtledove, and meadow bunting is mainly grain. The Formosan blue magpie is fed mice, meat, and fruit.

The fecal specimens were put into buffered glycerol saline and brought to the laboratory where they were plated on eosin-methylene-blue medium and Salmonella-Shigella agar. After an 18- to 24-hour incubation period, the colonies were tested by standard enteric methods and the results were tabulated (table 1).

RESULTS

Unlike fecal material from mammals, few bird specimens harbored *Escherichia coli*. This is also true of domestic fowl in which Aerobacter, Klebsiella, and intermediate coliforms take the place of Escherichia.[1] The group of paracolobactra showed some remarkable features. The feces of 1 plumed egret and of 1 cormorant harbored *Paracolobactrum aerogenoides* group 8,* while 2 snowy egrets had group 4 organisms. Other groups found were: group 11 in a blue magpie, group 12 in 1 plumed egret and 1 great egret, and group 15 in 1 great egret. *Paracolobactrum aerogenoides* groups 4 and 8 are frequently found in man, soil, vegetables, and domestic mammals. Serological study of the *Paracolobactrum coliforme* strains revealed group

* The serological groups of *P. aerogenoides* are numbered according to Eveland and Faber (Eveland, W. C., and Faber, J. E.: Antigenic Studies of a Group of Paracolon Bacteria. *J. Infect. Dis., 93,* [1953]: 226), those of *P. coliforme* according to Eveland *et al.* (Eveland, W. C., Felsenfeld, O., Babione, R. W., Kuhns, D. M., and Kase, A.: Further Studies of *Pc. Coliforme* Strains in the Far East. *U. S. Armed Forces M. J., 6,* [1955]: 1605).

(3,4,5)6,(9)† organisms in 1 black-crowned night heron, 1 snowy egret, and 1 cormorant. This group of paracolobactra includes some pathogenic strains. *Paracolobactrum coliforme* group 12 was isolated from 2 great egrets and 1 black-crowned night heron. These serotypes have been found in dogs and man. Evidently paracolobactra, frequently present in man, vegetables, and soils in Japan,[2] are also present in birds.

Many Providencia and transitional paracolon organisms were also isolated. Four of the 61 birds showed Providencia and 20 related transitional strains. Providencia and transitional forms are not rare in cats, dogs, and market meat in Japan. Providencia was found, however, in only 25 and transitionals in 15 out of 1,963 fecal specimens from human beings examined in the Far East in 1955. The transitional group of paracolobactra consists of bacteria which have reactions similar to those of Providencia but differs from them in being unable to utilize citrate as the sole source of carbon. These organisms are distinguishable from Proteus by their inability to form urease. It is possible that some members of this provisional group will be reclassified as Providencia or Erwinia. At present, according to the outlines of Bergey [3] and Kauffmann,[4] these organisms can not be put into any of the well-defined genera of Enterobacteriaceae. They did not deviate significantly from other groups of paracolobactra. No Salmonellae were isolated.

† These numbers refer to serological groups. Those in parentheses designate antigens which may or may not occur in a given group. This way of marking unstable antigens has been used for some time in Salmonellae.

DISCUSSION

A study of droppings collected from seven species of wild birds and six species of caged birds seems to indicate that the Enterobacteriaceae harbored by them may

TABLE 1—Occurrence of Enterobacteriaceae in Birds

				No. in which Enterobacteriaceae present												
							Paracolobactrum						Proteus		
Feeding	Nesting	Species	Group and No. examined	*Esch. coli*	Intermediate coliforms	*Esch. freundii*	*aerogenes*	Intermediate	*coliforme*	anaerogenic	Providencia	Transitional	*vulgaris*	*mirabilis*	*morgani*
In rice paddies	In colonies	Black-crowned night heron (*Nycticorax nycticorax*)	I	10	1	....	....	....	....	5	1	....	8	....	....	....
		Great egret (*Egretta alba*)		10	3	....	....	3	....	7	1	1	5	....	1	1
		Plumed egret (*Egretta intermedia*)		3	....	....	....	1	....	1	....	....	1	....	....	....
		Little egret (*Egretta garzetta*)		9	1	....	3	3	2	2	....	1	4	1	....	2
Group total				32	5	....	3	7	2	15	2	2	18	1	1	3
Forest, farmyards	Single nests in forests	Blue magpie (*Cyanopica cyanus*)	II	13	....	....	....	5	....	2	1	....	1	....	....	1
In ocean	Isolated islands	Streaked shearwater (*Puffinus leucomelas*)	III	2	....	....	....	....	1	....	....	....	....	....	....	....
In Tokyo Bay	In colonies	Cormorant *Phalacrocorax carbo*		7	....	1	....	3	....	2	....	....	1	1	....	1
Group total				9	....	1	....	3	1	2	....	....	1	1	....	1
Caged birds		Bobwhite quail (*Colinus virginianus*)	IV	2	1	1	....	....	....	....	....	....	....	....	....	....
		Emerald dove (*Chalcophaps indica*)		1	1	....	....	....	....	....	....	....	....	....	....	....
		Formosan blue magpie (*Kitta caerulea*)		1	....	....	....	....	....	1	....	....	....	....	....	....	1
		Meadow bunting (*Emberiza cioides*)		1	....	1	1	1	....	....	....	1	....	....	....	....
		Zebra dove (*Geopelia striata*)		1	1	....	....	....	....	....	....	....	....	....	....	....
		Turtledove (*Streptopelia orientalis*)		1	1	....	....	....	....	....	1	1	....	....	....	....
Group total				7	4	2	1	1	....	1	1	2	....	....	....	1
Total				61	9	3	4	16	3	20	4	4	20	2	1	6

originate from mammals. Caged birds and birds living free but near human habitations seem to acquire both human and animal bacteria.

The cause of the frequent occurrence of transitional paracolon organisms in these wild birds is not evident. Their pathogenic power is unknown. Providencia strains, many of which are considered pathogenic, and *P. coliforme* group 3,4 organisms which have been isolated from human feces are significant findings. Since none of the species of birds tested, except blue magpie, usually come in contact with fruits and vegetables consumed raw by man, the presence of these bacteria in the birds probably does not constitute a menace to human health. However, black-crowned night heron, blue magpie, meadow bunting, and turtledove are commonly hunted and eaten by man which might suggest a medium of infection. Since none of the birds tested appeared sick, it may be supposed that the paracolobactra isolated are not pathogenic for these species. Certain strains of bacteria may be increased by colonial nesting; of this group of birds, the night heron, egret, and cormorant were colonial.

Summary

Sixty-one specimens of bird droppings from 13 species of birds were examined for the occurrence of Enterobacteriaceae. The intestinal flora of these birds resembled that of man and domestic animals and was similar to bacteria found in soils and on vegetables. The results indicated that birds feeding near human habitations pick up human and animal Enterobacteriaceae.

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Studies on *Pasteurella Multocida*. II. Identification of Antigenic Characteristics and Colonial Variants

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IN THE FIRST REPORT[7] in this series, a hemagglutination test was described for the identification of serological types of *Pasteurella multocida*. Because the results of serological typing depend upon the presence or absence in cultures of a predominance of capsulated organisms, a method was sought for the identification of noncapsulated colonial variants of *Past. multocida*. This report describes the use of the acriflavine test for this purpose. This test has also aided in the identification of the principal variants, making possible the designation of variants by the same nomenclature that has been used for other gram-negative species.

The dissociation pattern of this species has been dealt with in detail in a number of reports.[10,12] The various equivalent terms used to designate variants have been grouped (table 2, column 1) and after each term is given the reference to the report in which the terms were first employed. It is evident that a standard nomenclature for the description of variation in *Past. multocida* is required.

The acriflavine test, first introduced by Allessandrini and Sabatueci,[1] has been employed by a number of workers for the identification of antigen characteristics of colonial variants. Braun [4] points out "that the acriflavine test has proved to be a far more reliable indication of antigen characteristics than mere observation of colony morphology." The reactions in acriflavine given by Braun are: Antigenically smooth cells remain suspended, antigenically rough cells yield clumps, and antigenically mucoid cells produce slimy precipitates. Because of the independent changes between antigenic and colonial characteristics, Braun recommends the designation of colonial characteristics as smooth, rough, and mucoid, and the designation of antigen characteristics by the symbols S, R, and M. This nomenclature will be employed in this report.

MATERIALS AND METHODS

Strains of Pasteurella Multocida Examined.—In excess of 200 strains representing all four serological types, A, B, C, and D, were examined by the acriflavine test. Four cultures chosen to illustrate typical reactions are listed (table 1, column 1).

Examination of Colonial Variants.—The strains examined were grown on tryptose agar to which had been added 5 per cent by volume of horse serum. The different variants were studied by means of obliquely transmitted light. This method described in detail by Braun has been used for a number of years for the recognition of colonial variants of Brucella species.

The term "fluorescence" has been used frequently to describe the interplay of colors of the spectrum around smooth colonies of *Past. multocida*. This term, although widely used, is incorrect and is replaced in this study by the correct term "iridescence" except where an author's original terminology is being employed.

Performance of the Hemagglutination Test.—The indirect hemagglutination test was conducted in the manner described previously.[7] In the first report on this test, it was pointed out that strong cross reactions were obtained with bovine serums when red cells were treated with the capsular substance from predominantly mucoid strains. This difficulty was overcome by the use of carefully prepared chicken and rabbit serums. A large number of predominantly mucoid strains have been typed satisfactorily by the hemagglutination test.

Performance of the Acriflavine Test.—The method employed was a modification of the microscopic test described by Braun and Bonestell.[3] It differed from their technique in that larger amounts of bacterial cells, saline, and acriflavine * solution were employed, thus giving macroscopically observable reactions. A 1:1,000 solution of acriflavine in distilled water was employed. The solution was stored in a brown bottle and discarded one week after preparation.

The test was carried out as follows: (1) A number of colonies were picked from the culture plate (blood agar or serum agar) with an inoculating loop and deposited in the center of a glass microscopic slide. (2) One or 2 drops of saline,

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* Acriflavine (neutral), N.F., National Aniline Division, Allied Chemical and Dye Corp.

TABLE 1—Representative Strains of Pasteurella Multocida Demonstrating the Relationship Between "Typeability," Colonial Morphology, and Reaction in Acriflavine

Strain and serological type	Colonial morphology	"Typeable" by hemagglutination	Acriflavine reaction	Antigenic designation
1 (chicken) A	Mucoid	Yes	Slimy	M
2 (buffalo) B	Smooth	Yes	Cells remain suspended	S
3 (dog) C	Smooth	No	Flocculation	S^R
4 (pig) D	Smooth	Yes	Coarse clumping	S

depending upon the size of the drop and the amount of growth, was placed over the deposit of bacterial cells which, with the inoculating loop, were mixed thoroughly with the saline. (3) One or 2 drops of the 1:1,000 acriflavine solution was added to the bacterial suspension. Mixing was then accomplished by rocking and tilting the slide. The reaction was apparent shortly after mixing.

If one is interested in only individual colonies, the microscopic test described by Braun may be employed. The macroscopic slide test described above is particularly useful as it indicates the antigenic characteristic and colonial variant that predominate in a given culture.

RESULTS

The reactions observed with the acriflavine test were essentially the same as those referred to by Braun as occurring with the variants of other gram-negative species.

Three principal colonial types were observed by obliquely transmitted light. They were: (1) Mucoid—these colonies were, comparatively speaking, the largest. They had flowing margins and had a characteristic moist appearance. As Elberg and Cheng-Lee Ho [10] pointed out, they produce a reddish fluorescence. Frequently, the "fluorescence" is only slight and the colonies have a white opaque fluffing appearance. (2) Smooth (iridescent)—these colonies are smaller and discrete. They display a marked iridescence which is usually greenish. (3) Smooth (noniridescent)—the organisms of these colonies possess less capsular substance than organisms constituting iridescent colonies. They are usually gray or grayish blue. They have sometimes been referred to as intermediate variants in the literature. The blue colonies are composed of noncapsulated organisms. Noniridescent colonies are discrete and smaller than the other colony types.

In table 1 are listed representative strains which show the relationship between colonial morphology, "typeability" by hemagglutination, acriflavine reaction, and antigen character. Only the smooth strain which gave the flocculent acriflavine reaction could not be typed. Cultures composed of mucoid variants gave a slimy or stringlike precipitate (fig. 1, A). Variants giving this reaction were designated as mucoid M—the word refers to colonial morphology, and the M indicates the antigen characteristic. Most of the freshly isolated cultures yielding predominantly mucoid variants were type A strains. On repeated transfer, the mucoid variants were replaced by smooth variants.

Two distinct reactions were observed with smooth variants. The cells of the iridescent smooth variants remained almost completely in suspension (fig. 1, B). Variants giving rise to this reaction were termed smooth, S. When iridescence was lost but capsular antigen was still present, as determined by the hemagglutination test, a partial flocculation was noticed in acriflavine. The same designations, smooth S, were employed. Morphologically smooth variants, not possessing capsular substance

TABLE 2—Proposed Colonial Designations Corresponding to Older Designations and the Reaction of the Different Variants with Acriflavine

Designations employed in the past	Proposed designations		Capsule	Reaction in acriflavine
	Colonial	Antigenic		
Mucoid [13]	Mucoid	M	++	Slimy precipitate
Fluorescent [11]				
Diffuse [8]	Smooth	S	+	Cells remain in suspension (except type D)
Smooth [2]				
Intermediate [11]	Smooth	S	+	Partial flocculation
Granular [8]	Smooth	S^R	−	Flocculation
Blue [11]				
Rough [2]	Rough	R	−	Flocculation

and yielding negative hemagglutination tests, gave a markedly flocculent or granular type of reaction (fig. 1, C). The variants giving rise to this reaction were designated smooth S^R, the exponent R indicating antigenic roughness.

Smooth colonies giving S^R reaction flocculate in saline in most instances. Another characteristic of antigenically rough cultures is their tenacious adherence to agar. It is only with great difficulty that the growth is washed from the surface of the agar. These cultures display a low virulence for mice.

The observations cited above refer to types A, B, and C. The smooth variants of freshly isolated type D strains gave a characteristic coarse clumping in acriflavine (fig. 1, D).

The results obtained with the acriflavine test are summarized (table 2). In columns 2 and 3 of table 2 are listed the designations which are proposed as a standard nomenclature for the description of the variants of *Past. multocida*.

Cultures whose variants are both morphologically and antigenically rough are rare.

The S^R cultures were a source of confusion for many years because, although their mouse virulence was often great, they did partake of some of the properties of rough cultures. In the literature, they have sometimes been referred to as rough, although morphologically they are usually smooth.

DISCUSSION

It has been shown that the capsular antigen of *Past. multocida* is strongly immunogenic.[5] The importance of selecting capsulated strains for the preparation of bacterins and antiserums is axiomatic. The acriflavine test offers a simple procedure for determining the antigenic status of cultures. Strains giving rough reactions

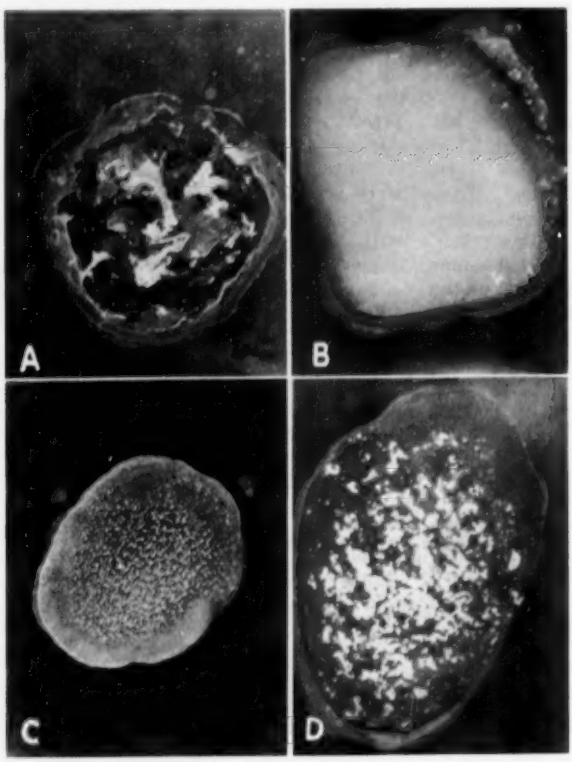

Fig. 1—Reactions occurring in suspensions of different variants of Pasteurella multocida after the addition of 1:1,000 neutral acriflavine.

A) Mucoid variants—slimy precipitate, (B) smooth variants—cells remain in suspension, (C) smooth variants but antigenically rough—flocculent precipitate, and (D) smooth variants, type D—coarse clumping.

certainly should not be employed. It is of interest that although many cultures lose their capsular antigen as a result of repeated transfer some strains are remarkably stable.

In a previous report, it was suggested that dissociation in this species proceeded, smooth ⟶ mucoid ⟶ rough. The observations made during this study suggest the sequence, mucoid ⟶ smooth ⟶ smooth (S^R). Confusion arose in the previous report because of the occurrence of many mucoid cultures with mouse virulence less than that of smooth cultures. It was considered that these cultures were composed of a special variant and it seemed reasonable to place this variant between the smooth and what was then termed the rough variant. A number of experiments [5] have shown that the delayed mortality pattern for mice of many mucoid cultures has been due to the action of symbiotic or latent bacteriophage. It is now realized that the lowered mouse virulence is a property of some mucoid cultures but not of all mucoid strains.

Summary

The colonial morphology and antigenic behavior of variants of *Pasteurella multocida* have been studied by means of obliquely transmitted light and the acriflavine test, respectively. It is proposed that the colonial variants of *Past. multocida* be designated by the standard terminology, smooth, mucoid, and rough, and that the antigenic characteristics be indicated by the letters S, M, and R. A characteristic reaction was observed when freshly isolated type D strains were examined with the acriflavine test. It is pointed out that besides aiding in the elucidation of variation in this species the test rapidly identifies nonimmunogenic cultures and those that can not be typed.

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The Effects of Some Antibiotics and Chemical Drugs on the Growth *in Vitro* of *Aspergillus Fumigatus*

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In a previous paper,[1] we described the first cases of aspergillosis in Greece in White Leghorn adults and chicks, 10 to 15 days old. The diseased chicks showed clinical symptoms of pneumonia, while the adults died without any apparent symptoms.

Pathological lesions, consisting of tubercle-like bodies of various sizes and colors and caseous in nature, were observed on the liver, lungs, gizzard, and cecum. The lesions yielded pure cultures of *Aspergillus fumigatus* mainly and, to a lesser extent, *Aspergillus niger*.

Intravenous and subcutaneous injections of conidia taken from the cultures reproduced the disease, whereas intratracheal instillation did not. Furthermore, the inoculation of embryonating chicken eggs, 13 to 17 days old, caused the death of the embryos.

The disease apparently resulted from use of moldy litter and feeds. When these were eliminated, the disease disappeared.

In this paper, we intend to report the results of our experiments on the sensitivity of *A. fumigatus in vitro* to the commonly used antibiotics and to two chemical drugs.

Materials and Methods

Aspergillus fumigatus was cultured in a liquid nutritive medium consisting of: beef extract (Difco), 6 Gm.; bacto peptone (Difco), 15 Gm.; sodium chloride, 5 Gm.; glucose, 30 Gm.; and distilled water, 1,000 ml., with a final pH of 6.5.

The hanging drop method was used[4] as follows: A rubber ring, spread with vaseline,® was applied to a slide, delimiting in that manner a chamber; 2 to 3 drops of the nutritive medium, inoculated with a large number of conidia, were put in this chamber; and a second slide was applied against the rubber ring, thus closing it. The whole was placed on a tray and put in a wide-mouthed bottle containing a small quantity of water, to prevent drying of the preparations. The bottle was then covered with a glass bell and placed in an incubator at 37 C.

A rich growth of *A. fumigatus* was observed 24 hours later. The color of the colonies was yellowish green in the beginning, becoming dark green when the fruiting bodies were fully formed.

The following antibiotics and chemical drugs were tested: penicillin (specilline, Specia), the rate of which was calculated on the basis of 1,650 I.U. equals 1 mg.; streptomycin (didromycine, Specia); aureomycin® and achromycin® (Lederle); terramycin® (Pfizer); chemicetine (chloramphenicol, Erba); chloromycetin® (chloramphenicol, Parke, Davis); erythromycin (erythrocin,® Abbott); potassium iodide; and mercuric iodide red (Merck). These substances were diluted in the liquid culture medium in various concentrations; control slides were also prepared. The preparations were examined after 24 hours of incubation.

Results

The results of our experiments are shown (tables 1 and 2).

Discussion

To date, no attempt has been made to establish the effectiveness of the commonly

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TABLE 1—Results of Testing Eight Antibiotics in Various Concentrations Against Aspergillus Fumigatus in Vitro

Antibiotics	Controls	Antibiotic concentration per milliliter of culture medium							
		62.5 μg.	125.0 μg.	250.0 μg.	500.0 μg.	1.0 mg.	2.0 mg.	5.0 mg.	10.0 mg.
Penicillin	+++	+++	+++	+	+	+	+	+	+
Streptomycin	+++	+	+	+	+	+	+	+	+
Aureomycin	+++	+	+	+	+	+	+	+	+
Achromycin	+++	+	+	+	+	+	+	+	+
Terramycin	+++	+	+	+	+	+	+	+	+
Chemicetine	+++	+	+	+	+	+	+	+	+
Chloromycetin	+++	+	+	+	+	+	+	+	+
Erythromycin	+++	+	+	+	+	+	+	+	+

+++ = germination and abundant fructification; + = germination without fructification.

TABLE 2—Results of Testing Two Chemical Drugs in Various Concentrations Against Aspergillus Fumigatus in Vitro

Chemical drugs	Controls	Drug concentration per milliliter of culture medium									
		3.125 μg.	6.25 μg.	9.37 μg.	12.5 μg.	18.75 μg.	25.0 μg.	37.5 μg.	50.0 μg.	100.0 μg.	10.0 mg.
Potassium iodide	+++	+	+	+	+	+	+	+	+	+	+
Mercuric iodide red	+++	+	+	+	+	±	−	−	−	−	−

+++ = germination and abundant fructification; + = germination without fructification; ± = light germination; − = neither germination nor fructification.

used antibiotics against *A. fumigatus in vitro*. Our experiments show that:

1) Penicillin is inactive against *A. fumigatus* when used in concentrations up to 125.0 μg. per milliliter of the culture medium. It is, however, in higher concentrations, fungistatic in that it inhibits fructification.

2) The other antibiotics used, as well as potassium iodide, are only fungistatic in concentrations up to 10.0 mg. per milliliter.

3) Mercuric iodide red is fungistatic in amounts up to 18.75 μg. per milliliter but, above this concentration, it is markedly fungicidal.

These results obtained *in vitro* do not seem to be in agreement with those of Mantovani and Airoldi,[2] who claim to have treated infected chickens successfully by using streptomycin. Horton-Smith and Long[3] have shown that penotrane (phenylmercuric-dinaphthylmethane-disulfonate) has a stronger fungicidal effect (diluted 1:80,000) than mercuric iodide red (diluted 1:40,000).

Summary

1) Penicillin was found to be totally inactive against *Aspergillus fumigatus in vitro* in concentrations up to 125.0 μg. per milliliter of culture medium; it showed a fungistatic effect at 250.0 μg. per milliliter or higher.

2) The seven other antibiotics tested—streptomycin, aureomycin,® achromycin,® terramycin,® chemicetine (chloramphenicol, Erba), chloromycetin® (chloramphenicol, Parke, Davis), and erythromycin—and potassium iodide have been proved fungistatic in concentrations up to 10.0 mg. per milliliter.

3) Mercuric iodide red has been proved to be fungistatic in concentrations up to 18.75 μg. per milliliter and fungicidal above this concentration.

Further experiments are in progress concerning the use of mercuric iodide red *in vivo* against aspergillosis of chickens.

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Observations of the Time of Appearance of Negri Bodies, Complement-Fixing Antigen, and Virulence in Mouse Brains Inoculated with Rabies Street Virus

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FOR the routine diagnosis of rabies in animals, this laboratory has simultaneously used three techniques, *viz.*, demonstration of (1) Negri bodies in impression preparations and in histological section, (2) specific rabies complement-fixation antigen, and (3) viable rabies virus in the suspect brain. Usually, the findings by the three techniques have been found to supplement each other but, in some cases, neither Negri bodies nor complement-fixing antigen were found despite later isolation of virus by inoculation of mice. This lack of agreement may be due to early destruction of the suspect animal at a time when neither Negri bodies nor antigen were demonstrable. In general, findings by the complement-fixation procedure have more frequently been in agreement with isolation results than have results of searches for Negri bodies. The following experiments were designed to determine the time after infection when positive findings by each of the techniques could be expected.

MATERIALS AND METHODS

Procedures described by Ando *et al.*[5,6] were slightly altered by us. Our procedures were as follows. Antigen was made by placing a 20 per cent saline emulsion of infected or suspect brain in a 40-C. water bath for 60 minutes and then centrifuging at 12,000 r.p.m. for 15 minutes. The supernatant fluid was removed and used as antigen. Known positive serum was obtained by bleeding guinea pigs which had been hyperimmunized by four intraperitoneal injections of ultraviolet-inactivated, fixed (MDH strain) rabies virus. Each injection consisted of 2.0 ml. of 10 per cent guinea pig brain and was given at weekly intervals. This serum was inactivated immediately before use in the test by holding for 20 minutes in a 60-C. water bath. Two full units of complement contained in 0.3 ml. were used. Sensitized cells were prepared by combining equal volumes of 3 per cent washed sheep erythrocytes and hemolytic amboceptor containing 3 minimal hemolytic units in 0.15 ml.

The actual test was performed by combining 0.15 ml. of inactivated, hyperimmune serum in serial twofold dilutions with 0.15 ml. of undiluted antigen and 0.3 ml. of complement containing 2 full units. These were incubated at 37 C. for 120 minutes, after which 0.3 ml. of sensitized cells were added. The test was incubated at 37 C. for 30 minutes and then the reactions were recorded. Complete hemolysis was recorded as 0, absence of hemolysis as 4, with 1, 2, and 3 indicating 75, 50, and 25 per cent hemolysis, respectively. A 2+ or greater fixation was recorded as a positive.

Ten per cent emulsions of four known rabies-positive dog brains in physiological saline solution were each centrifuged at 2,000 r.p.m. for 15 minutes. Then 0.03 ml. of each supernatant was inoculated intracerebrally into about 30, 10- to 11-Gm. normal mice. Beginning on postinoculation day 4, 2 or 3 mice were killed daily. Brains of these mice were removed under aseptic conditions and then longitudinally divided. One half of each brain was fixed for sectioning, to be later stained with Mann's stain[1] or hematoxylin-eosin. The other halves were pooled and made into a 20 per cent emulsion in physiological saline solution. A part of this emulsion was used for titration of virus infectivity, inoculating groups of 5, 10- to 11-Gm. mice with serial tenfold dilutions from 10^{-1} through 10^{-6}. These inoculated animals were

TABLE 1—Time of Appearance of Infectivity, Specific Rabies Complement-Fixing Antigen, and Negri Bodies in Mice After Inoculation with Four Strains of Rabies Virus

Virus strain	First day virus isolated	First day mice CF positive	First day Negri bodies identified
1	9	10	12
			12
2	4	6	7
			7
3	6	9	10
			11
			12
4	6	8	9
			9
			10
Mean	6.25	8.25	9.9

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observed daily for four weeks, at the end of which time the infectivity expressed in $l.d._{50}$ was calculated after the method of Reed and Muench.[3] The remainder of the 20 per cent mouse brain suspension was used in preparation of complement-fixing antigen.

RESULTS

Virus from Dog 1.—Highly suggestive intracytoplasmic inclusion bodies were seen in brains of mice killed on the eleventh postinoculation day. Typical Negri bodies were observed in brains of mice killed on the twelfth day and later. Specific rabies complement-fixing antigen was first detected on postinoculation day 10. In the case of this virus, no titration was made on days 4, 5, 6, or 8, but the $l.d._{50}$ of virus in the brains harvested on day 9 was $10^{-1.47}$. The $l.d._{50}$ then increased rapidly, so that on day 12 and after it was $10^{-5.5}$ or greater.

Virus from Dog 2.—Typical Negri bodies were observed from day 7 until the death of all inoculated mice. Specific complement-fixing antigen was first detected on day 6. The virus titer was first measured on day 4 when the $l.d._{50}$ was $10^{-1.31}$. This increased slowly until day 9 when it was greater than $10^{-5.5}$.

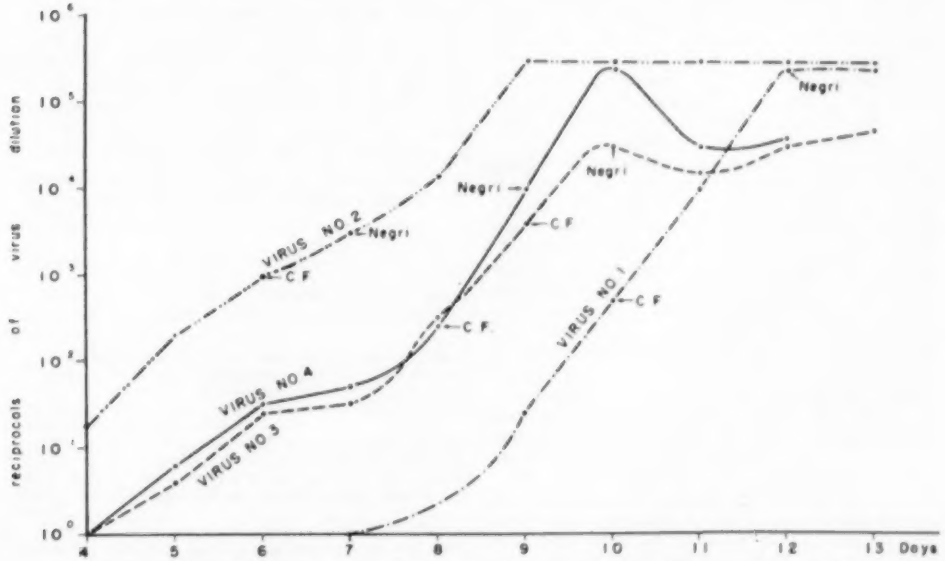
Virus from Dog 3.—Three mice were killed daily from the group inoculated with this dog brain. Typical Negri bodies were seen in 1 of the 3 mice killed on days 10 and 11. In the other 2 mice killed on these days, only a nonbacterial encephalitis was seen. From day 12 on, the complement-fixation reaction was positive. Virulence increased from an $l.d._{50}$ of $10^{-1.48}$ on day 6 to $10^{-3.7}$ on day 9 and $10^{-4.5}$ on day 10. On day 13, the $l.d._{50}$ was $10^{-4.7}$.

Virus from Dog 4.—Three mice were killed daily from this group of mice. Typical Negri bodies were present in 2 of the 3 mice killed on day 9. The other mouse brain was observed to have only a nonbacterial encephalitis. From day 10 on, Negri bodies were seen in every brain examined. Complement-fixing antigen was demonstrated after day 8. Virulence increased from an $l.d._{50}$ of $10^{-1.56}$ on day 6 to $10^{-5.5}$ on day 10, after which virulence noticeably decreased.

The time of appearance of (1) infectivity, (2) specific rabies complement-fixing antigen, and (3) Negri bodies in the case of four strains of street rabies virus is illustrated (table 1 and graph 1).

CONCLUSIONS

1) In point of time after infection, via intracerebral inoculation of mice, the three diagnostic procedures are reliable in the



Graph 1—Growth of four strains of rabies virus in mouse brain with appearance of Negri bodies and detectible complement-fixing antigen.

following order. (1) virus isolation, (2) demonstration of complement-fixing antigen, and (3) demonstration of Negri bodies. One or two days was observed as the interval between appearance of each in this series.

2) These data re-emphasize the importance of permitting rabies-suspect animals to proceed to death under observation rather than destroying them at a time when neither Negri bodies nor complement-fixing antigen would be present.

3) Complement-fixing antigen did not appear until the intracerebral titer had reached the level of $10^{-2.4}$ to $10^{-3.8}$ mouse $l.d._{50}$ per 0.03 ml.

4) Negri bodies did not appear until several days (7 to 12) had elapsed after injection or after a titer of $10^{-3.4}$ to $10^{-5.5}$ had developed, or both.

5) Rather wide variations in virulence, time of appearance of complement-fixing antigen, and Negri bodies were observed in the case of each of the four rabies isolates studied.

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Some Effects of Various Environmental Conditions on the Survival of *Leptospira Pomona*

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ALTHOUGH the economic and public health significance of leptospirosis caused by *Leptospira pomona* has been recognized, as yet there is very little information concerning the survival of this organism outside the animal body.

Ward and Turner [11] demonstrated the presence of *Leptospira icterohaemorrhagiae* for the period of two months in a well used as a source of drinking water, while van Thiel [10] reported that this organism can remain alive and completely virulent for at least 22 days. Reinhard [6] reported that *L. pomona* can persist in natural waters for several weeks. Noguchi [5] found that polluted water, sewage, or soil would not serve to keep *L. icterohaemorrhagiae* alive for more than three days, and that the organisms were unable to survive in human urine unless the urine was neutralized or slightly alkalinized. Burnstein and Baker [2] reported that urines of infected swine with a pH 7.0 or above usually contained active *L. pomona*, while in those below pH 7.0 the organisms appeared inactive but were nevertheless viable. Chang *et al.*,[3] in their studies with *L. icterohaemorrhagiae*, found that the survival times varied considerably depending upon the pH, temperature, and the density of other bacterial populations in the suspending fluids.

The following investigation was made in an attempt to determine the length of time *L. pomona* can remain infective outside the animal body.

MATERIALS AND METHODS

Only one strain of *L. pomona*, obtained from the Rocky Mountain Laboratory, Hamilton, Mont., was used throughout these experiments. This organism has been maintained on artificial mediums for several years and was selected because its virulence was assumed to be stabilized. The medium used throughout these experiments was a modification of Gardner's medium,[7] and all subcultures for the detection of viable leptospiras were held for 28 days at room temperature (20 to 26 C.) before being discarded. All microscopic examinations were made using dark field illumination. Weanling mice (Webster Swiss) were used when laboratory animals were required.[8]

Effect of pH and Temperature.—To determine the survival times of *L. pomona* after it was exposed to various pH and temperature levels, a preliminary experiment was made. It showed that a pH below 6.0 or above 8.4 rapidly killed these organisms, and temperatures above 40 C. caused death with rapid lysis. Therefore, all subsequent experiments on the effect of pH and temperature were carried out using replicate tubes of double-distilled water buffered to pH 6.0, 7.2, and 8.4,[1] and held at the following temperature: 34 to 36 C., 20 to 26 C., 7 to 10 C., 2 to −2 C., and −20 C. These tubes were inoculated so as to have approximately 2,000,000 leptospiras per milliliter.

At periodic intervals, a loopful was examined microscopically and 0.1-ml. quantities were cultured in semisolid mediums. In order to determine the effect of any serum which may have been carried over with the inoculum, one set of tubes was inoculated with leptospiras obtained by repeated centrifugations and washing three times with double-distilled water buffered at pH 7.2.

Effect of Moisture.—The effect of moisture content in soil was determined in the following manner: Replicate sets of tubes, each containing 2.0 Gm. of soil (pH 6.9), were sterilized by steam under pressure. One set was allowed to dry; to the second, sterile double-distilled water was added to a point of dampness; to the third set, sterile double-distilled water was added to a point beyond saturation where water covered the soil. The tubes were inoculated with approximately 10,000,000 leptospiras from an actively growing culture and were held at room temperature. As evaporation occurred in the damp and supersaturated soils, additional water was added to maintain approximately the same moisture level throughout the holding period.

At periodic intervals, depending upon the degree of moisture content, 10.0 ml. of double-distilled water buffered to pH 7.2 was added to one tube from each set to bring the soil into suspension. A loopful was examined microscopically

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for motile organisms, and 0.1 ml. inoculated into semisolid mediums. The effect of drying on this organism was also determined by placing a drop of culture on sterile slides and allowing it to dry. At two-minute intervals the slides were placed in tubes of liquid mediums.

Effect of Simulated Natural Conditions.—To determine the survival and infectivity of *L. pomona* under simulated natural conditions, and in the presence of natural microorganisms, a stainless steel rectangular tank was filled with a clay-type soil. At various space intervals, four pools (No. 1 to 4) were connected in series by narrow channels, pool 1 being closest to the water source and pool 4 emptying into a jar of disinfectant. A fifth pool was connected to pool 2 and located in such a position as to create a stagnant condition. The amount of flow was regulated by a valve at the intake.

The water used in these experiments was collected from a small, slow-moving stream which flowed through a pasture periodically inhabited by cattle. *Leptospira pomona* had been isolated from this stream in 1954.[4] Water was replenished frequently at the influent to maintain a relatively fresh flow through the system. All water samples used in these experiments were found to be free of pathogenic leptospiras through mouse inoculation and microscopic examination. The water was pH 7.8, and no significant change in pH occurred during the experimental periods.

Four experiments were carried out using this simulated natural water supply. In each experiment, approximately 300,000,000 organisms were used as the inoculum. Samples were examined at periodic intervals both microscopically and by mouse inoculations. In the first series of experiments, the flow of water was regulated to 4.6 ml. per minute (at this rate there occurred a volume turnover in 2.5 hours), and the organisms were introduced into pool 1. In the second series of experiments, the flow of water was regulated so as to equal evaporation and maintain original levels in each pool, and the organisms were introduced into pool 5. In the second experiment of each series, cow manure was placed in pools 2 and 5 to increase the concentration of other microorganisms.

In an attempt to determine the influencing factors concerned with the survival of *L. pomona* in river water,* the following menstruums were used: nonsterile river water, river water sterilized by filtration, river water sterilized by autoclaving, and sterile double-distilled water buffered to pH 7.2. These suspending fluids, in 10.0-ml. quantities, were placed in sterile tubes and inoculated with an actively growing culture of *L. pomona*. All tubes were held at room temperature (20 to 26 C.). At various time intervals, a loopful was examined microscopically for motility; 0.1 ml. was inoculated into a tube of culture medium and a 0.2-ml. quantity was injected intraperitoneally into each of 2 weanling mice.

RESULTS

Effect of pH and Temperature.—Both pH and temperature markedly affected the survival of *L. pomona* (table 1). The critical levels were between pH 6.0 and 8.4. Below or above these levels, the leptospiras were rapidly rendered nonmotile (within 8 hours at pH 5.5 and within 2 hours at pH 8.8, when held at 20 to 26 C). At the —20 C. temperature level, the organisms remained motile for less than four hours after freezing. However, lysis of the cells did not occur until after a prolonged exposure period, whereas at 44 to 46 C. lysis occurred soon after cessation of motility. At the freezing temperatures, experimental evidence indicated that death was due to temperature and not pH, since no significant differences were observed at the various pH levels. However, when the organisms were held at 7 to 10 C., 20 to 26 C., and 34 to 36 C., pH did affect their survival times based on both motility and cultural observations. In the lower pH ranges, the organisms remained motile for longer periods at 7 to 10 C. than was observed at 20 to 26 C. In the higher pH ranges, the observed motility was considerably longer at 20 to 26 C. than at 7 to 10 C.

* River water for this experiment was obtained from the Palouse River, Pullman, Wash.

TABLE 1—Survival Times of Leptospira Pomona at Various pH and Temperature Levels

	pH 6.0				pH 7.2				pH 8.4			
	Unwashed		Washed		Unwashed		Washed		Unwashed		Washed	
Temperature	Mot.[a]	Cult.[b]	Mot.	Cult.	Mot.	Cult.	Mot.	Cult.	Mot.	Cult.	Mot.	Cult.
34 to 36 C.	2.0	1.0	0.5	0.5	6.8	6.5	2.2	2.0	2.4	2.0	2.0	2.0
20 to 26 C.	11.3	4.0	0.8	0.25	34.8	29.0	22.0	17.2	17.0	15.0	9.5	7.0
7 to 10 C.	12.2	8.0	3.5	4.2	54.0	44.5	18.0	12.0	2.6	2.0	0.5	0.5
2 to —2 C.	0.23	0.96	0.09	0.28	0.92	1.35	0.55	1.0	0.42	1.35	0.32	0.61
—20 C.	0.08	0.04	0.04	0.04	0.08	0.08	0.04	0.04	0.08	0.08	0.04	0.04

[a] Survival in days based on motility as observed by dark field illumination. [b] Survival in days based on growth in subculture incubated at room temperature for 28 days.

In all cases, unwashed cells survived for longer periods than did the washed cells, based on both motility and cultural studies. The survival times of unwashed leptospiras at pH 7.2 and temperature of 7 to 10 C. were found to be 54.0 days, as based on motility and 44.5 days when based on reproducibility. When washed cells were employed under these same conditions, the survival times were 18 and 12 days, respectively. The concentration of serum in the tubes containing unwashed cells was calculated to be 0.06 per cent.

TABLE 2—Maximum Time of Observed Motility and Cultural Detection of Leptospira Pomona, Held at 20 C. in Sterile Soil of Varying Water Content

Soil condition	Motility [a]	Culture [b]
Dry	0 hours	2 hours
Damp [c]	3 days	5 days
Supersaturated [d]	193 days	183 days

[a] Motility based on dark field observation. [b] Culture examined after 28 days of incubation at room temperature. [c] Sterile water, 2.0 ml., added to 2.0 Gm. of dry soil. [d] Sterile water, 6.5 ml., added to 2.0 Gm. of dry soil.

In nearly every case where survival of the organisms was prolonged beyond five to six days, their survival times based upon subculturing were less than when based on motility. On the other hand, when survival times were less than two to three days, the subcultures were often positive for extended periods beyond observed cessation of motility.

Effect of Moisture.—Under dry soil conditions, the leptospiras could not be detected microscopically after 30 minutes of exposure or culturally after two and a half hours (table 2). When exposed to damp soil conditions, the organisms could not be detected microscopically after three days and culturally after five days. However, under supersaturated soil conditions, the leptospiras were observed to be motile for 193 days and culturally positive for 183 days. When the organisms were dried on sterile glass slides, they could not be detected microscopically or by subculturing seconds after the slides were dry.

Effect of Simulated Natural Conditions.—Leptospira pomona, under simulated natural conditions, were detectable microscopically for 14 days but by mouse inoculation for only a maximum of six days. In the first series of experiments (table 3, series A, experiments 1 and 2), the leptospiras were observed microscopically in all pools within 20 minutes. In all experiments, under the stagnant conditions of pool 5, the leptospiras were detected by mouse inoculation for periods ranging from three to six days, while in other pools the organisms were detected for periods ranging from one and a half to two days.

A marked difference was noticed when comparing the survival times of *L. pomona* in nonsterile, Seitz-filtered, or autoclaved river water, and in sterile double-distilled water buffered to pH 7.2 (table 4). In nonsterile river water, leptospiras were detected microscopically for eight days, and by mouse inoculation for ten days. In Seitz-filtered river water, motility persisted for 99 days and cultures were positive for 94 days, while in autoclaved river water the organisms remained motile and viable for only 47 days. In sterile double-distilled water the leptospiras were detected microscopically for 18 days and by mouse inoculation for nine days.

DISCUSSION

The optimum temperature for survival of *L. pomona* seems to be between 7 C. and 26 C., depending upon the pH of the sus-

TABLE 3—Survival Times of Leptospira Pomona Under Simulated Natural Conditions

Experimental series	Pool 1		Pool 2		Pool 3		Pool 4		Pool 5	
	Mot.[a]	Inf.[b]	Mot.	Inf.	Mot.	Inf.	Mot.	Inf.	Mot.	Inf.
Series A [c]										
Experiment 1	14	<2	8	<2	13	<2	14	<2	14	4
Experiment 2 [d]	....	....	4	1.5	2	2	....	....	5	6
Series B [e]										
Experiment 1	Neg.	Neg.	3	2	....	....	....	....	4	3
Experiment 2 [d]	....	....	Neg.	Neg.	....	....	....	....	3	3

[a] Survival in days based on motility as observed by dark field examination. [b] Survival in days determined by infections in mice based on the demonstration of a rising blood serum antibody titer. [c] Current; 4.6 ml./minute = volume turnover in two and a half hours; unwashed organisms placed in pool 1 and found in all pools within 20 minutes. [d] Small quantities of cow manure placed in pools 2 and 5. [e] No current; flow to equal evaporation. Unwashed organisms placed in pool 5.

pending medium. The leptospiras survived for longer periods at 7 to 10 C. in the lower pH ranges than in the higher pH ranges and, conversely, for longer periods at 20 to 26 C. in the higher pH ranges. This indicated an interaction between temperature and pH when the organisms were held under these conditions. At freezing temperatures, no significant differences in survival times were observed at the various pH levels. This may be due to the fact that death occurred so rapidly in all cases that no observable effect of pH could be demonstrated. On the other hand, cultural results indicated that at freezing temperature the organism, although observed to be nonmotile, was nevertheless viable. This suggests that the organisms, for a short time at least, were in a dormant resting state with no energy being used for motility and when they were subcultured they were reactivated.

TABLE 4—Comparison of the Survival Times of Leptospira Pomona in River Water

Suspending fluid [a]	Motility [b]	Culture [c]	Infection [d]
River water [e]	8 days	.....	10 days
Filtered river water [f]	99 days	94 days	<18 days
Autoclaved river water [g]	47 days	47 days	.....
Double-distilled water [h]	18 days	.....	9 days

[a] All suspending fluids incubated at room temperature (20 to 26 C.). [b] Survival based on motility as observed by dark field illumination. [c] Survival based on growth in subculture, incubated at room temperature for 28 days. [d] Infection based on the demonstration of rising blood serum antibody titer in mice. [e] River water, nonsterile; pH 7.8 at start of experiment and 8.0 at the termination of the experiment. [f] River water autoclaved (121 C. for 30 minutes); pH 7.8 and no change. [g] River water filtered with Seitz type 1 filter; pH 7.8 and no change. [h] Double-distilled water autoclaved (121 C. for 30 minutes); pH 7.2 and no change.

That small amounts of serum can influence the survival time of these organisms was shown by comparing washed leptospiras with leptospiras obtained directly from culture mediums. The serum transferred with the inoculum increased the survival time by 32 days. Chang *et al.*[3] found that 0.1 per cent tryptose would increase the survival times of *L. icterohaemorrhagiae* by 20 days, while the addition of 1.0 per cent horse serum increased survival by 70 days.

Usually, where motility was observed for prolonged periods, the organisms appeared to lose their ability to propagate when subcultured. This seemed to be related to the length of time of exposure, since whenever their motility was observed for periods longer than five to six days, their propagating ability was proportionately decreased.

The prolonged survival of *L. pomona* in supersaturated sterile soil indicates that some factor in sterile soil was responsible for this longevity, since in neither double-distilled water nor in filtered or autoclaved river water did the organisms remain viable for such extended periods.

The rapidity with which *L. pomona* moved downstream under simulated natural conditions indicated that the spread of these spirochetes, in distance from the original source, could be great. On the other hand, it was indicated that fast-moving waters were detrimental to the survival of this organism. In one instance, leptospiras were observed and detected in pool 2, which was upstream from the point of inoculation. This may have been caused by a backwash as a result of disturbances of daily sampling in pool 5, or it suggests that leptospiras could migrate up a slow-moving stream, but for only relatively short distances.

Since the addition of small quantities of manure did not significantly shorten or prolong the survival times of *L. pomona* as determined by mouse inoculation, the prolonged observation of motile leptospiras in the first experiment might be attributed to the ease of detection due to the relatively clear water present. Under the conditions of the other experiments, where concentrations of other microorganisms and organic materials increased, the leptospiras may have been obscured and not readily detected microscopically. The difference in survival time observed in stagnant water, with and without fresh water flow, suggests that some factor, or factors, which prolongs the survival of *L. pomona* may be present in fresh water. This may be due to the oxidation-reduction potential, as indicated by Chang *et al.*,[3] a dilution of toxic materials, or some other factor.

Microorganisms naturally present in river water may have an inhibitory effect on the leptospiras, since the survival of *L. pomona* was considerably shorter in nonsterile river water as compared with sterile river water. The differences observed in Seitz-filtered and autoclaved river water may be due to the fact that, upon heating, some naturally occurring nutrient material or protective substance was destroyed. This view is further strengthened by the observation that in sterile double-distilled water the or-

ganisms became nonmotile and nonviable within a considerably shorter time.

SUMMARY

The survival of *Leptospira pomona* outside the animal body seems to be related to an interaction of several factors, including temperature, pH, moisture, the various constituents of soil and water, and the presence of naturally occurring microorganisms.

Temperatures below 7 to 10 C. or above 34 to 36 C. and pH below 6.0 or above 8.4 were detrimental to the survival of *L. pomona*. It survived longer at 7 to 10 C. in the lower pH ranges than in the higher and, conversely, for longer periods at 20 to 26 C. in the higher pH ranges.

Leptospira pomona was susceptible to both drying and slow-freezing—survival not exceeding one-half hour in the former and two and a half hours in the latter.

Moisture was extremely important, these organisms surviving from 30 minutes in air-dried soil to 183 days in soil supersaturated with water.

Leptospira pomona survived for less than seven days under simulated natural conditions and longer in stagnant pools than in moving water. However, the continuous addition of small amounts of fresh water to stagnant pools increased the organism's survival time.

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